PROJECT COMPLETION REPORT (PCR)

UNIVERSITY GRANTS COMMISSION
WESTERN REGIONAL OFFICE
GANESHKHIND, PUNE – 411 007.

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

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3. UGC APPROVAL LETTER NO. AND DATE : **47-870/13(WRO)** Dated **17/10/2014**

4. DATE OF IMPLEMENTATION: **11/02/2015**
5. TENURE OF THE PROJECT : **11/02/2015 to 10/02/2017** (Two Years)

6. TOTAL GRANT ALLOCATED : **285000/-**
7. TOTAL GRANT RECEIVED : **220000**/-
8. FINAL EXPENDITURE : **230279/-**

9. TITLE OF THE PROJECT **: to study the effect of stress degradation conditions on environmental sensitive pharmaceutical drugs by using chromatographic and spectroscopic technique**

10. OBJECTIVES OF THE PROJECT :

1. **To develop UV-Spectrophotometric methods for estimation of Cefdinir in bulk drug.**
2. **To develop High Performance Liquid Chromatographic method for estimation of in Cefdinir bulk drug.**
3. **To develop High Performance Thin Layer Chromatographic method for estimation of Cefdinir in bulk drug.**
4. **To isolate degradant by using Flash Chromatography method for estimation of cefdinir in bulk drug.**
5. **To characterization of isolated compound.**

11. WHETHER OBJECTIVES WERE ACHIEVED: **Yes**
12. ACHIEVEMENTS FROM THE PROJECT: Publication (in process)

13. SUMMARY OF THE FINDINGS :
(IN 500 WORDS) **Separate sheet attached**

14. CONTRIBUTION TO THE SOCIETY : **Patient compliance(Product will available in low cost for treatment of diseases)**

15. WHETHER ANY PH.D. ENROLLED/PRODUCED OUT OF THE PROJECT: **Nill**
16. NO. OF PUBLICATIONS OUT OF THE PROJECT: 02 (in process)

Dr V V Chopade
(Principal Investigator)

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PROJECT COMPLETION REPORT (PCR)

SUMMARY OF THE FINDINGS

Introduction [1- 14]

There is an ever increasing interest in impurities present in API's. Recently, not only purity profile but also impurity profile has become essential as per various regulatory requirements. In the pharmaceutical world, an impurity is considered as any other organic material, besides the drug substance, or ingredients, arise out of synthesis or unwanted chemicals that remains with API's.

The impurity may be developed either during formulation, or upon aging of both API's and formulated API's in medicines. Impurity profiling (i.e., the identity as well as the quantity of impurity in the pharmaceuticals), is now gaining critical attention from regulatory authorities.

Definition of Impurity Impurity is something that impure or makes something else impure. An impure substance may be defined as a substance of interest mixed or impregnated with an extraneous or usually inferior substance.

Impurity profile

Impurity profiling is a group of analytical activities, with the aim of detection, identification/structure elucidation and quantitative determination of organic and inorganic impurities, as well as residual solvents in bulk drugs and pharmaceutical formulations.

FORCED DEGRADATION STUDY

Forced degradation is the process of subjecting drug compounds to extreme chemical and environmental conditions to determine product breakdown levels and preliminary degradation kinetics, and to identify potential degradation products.

They are used to facilitate the development of analytical methodology, to gain a better understanding of active pharmaceutical ingredient (API) and drug product (DP) stability, and to provide information about degradation pathways and degradation products.

Forced degradation studies show the chemical behaviour of the molecule which in turn helps in the development of formulation and package. These studies are a regulatory requirement and scientific necessity during drug development, it is not considered as a requirement for formal stability program.

PROJECT COMPLETION REPORT (PCR)

Objective of forced degradation studies

1. Determination of degradation pathways of drug substances and drug products.
2. Discernment of degradation products in formulations that are related to drug substances versus those that are related to non-drug substances (e.g. excipients)
3. Structure elucidation of degradation products.
4. Determination of the intrinsic stability of a drug substance molecule in solution and solid state.
5. To reveal the degradation mechanisms such as hydrolysis, oxidation, thermolyis or photolysis of the drug substance and drug product.
6. To establish stability indicating nature of a developed method.
7. To understand the chemical properties of drug molecules.
8. To generate more stable formulations.
9. To produce a degradation profile similar to that of what would be observed in a formal stability study under ICH conditions.
10. To solve stability-related problems.

Degradation conditions

1. Hydrolytic condition
    - Acidic [HCL]
    - Basic [NaOH]
2. Oxidative condition
3. Thermal condition
4. Photolytic condition

1. Hydrolytic condition

Hydrolysis is one of the most common degradation chemical reactions over a wide range of pH. Hydrolysis is a chemical process that includes decomposition of a chemical compound by reaction with water. Hydrolytic study under acidic and basic condition involves catalysis of ionisable functional groups present in the molecule. Acid or base stress testing involves degradation of a drug substance by exposure to acidic or basic conditions which generates primary degradants in desirable range. The selection of the type and concentrations of acid or base depends on the stability of the drug substance. Hydrochloric acid (HCL) or

sulphuric acids (0.1–1 M) for acid hydrolysis and sodium hydroxide or potassium hydroxide (0.1–1 M) for base hydrolysis are suggested as suitable reagents for hydrolysis. If the compounds for stress testing are poorly soluble in water, then co-solvents can be used to dissolve them in HCl or NaOH. The selection of co-solvent is based on the drug substance structure. Stress testing trial is normally started at room temperature and if there is no degradation, elevated temperature (50^{o}–70°C) is applied. Stress testing should not exceed more than 7 days. The degraded sample is then neutralized using suitable acid, base or buffer, to avoid further decomposition.

2. Oxidative condition

Hydrogen peroxide is widely used for oxidation of drug substances in forced degradation studies but other oxidizing agents such as metal ions, oxygen, and radical initiators (e.g. azobisisobutyroni-trile, AIBN) can also be used. Selection of an oxidizing agent, its concentration, and conditions depends on the drug substance. It is reported that subjecting the solutions to 0.1–3% hydrogen per-oxide at neutral pH and room temperature for seven days or up to a maximum 20% degradation could potentially generate relevant degradation products.

The oxidative degradation of drug substance involves an electron transfer mechanism to form reactive anions and cations. Amines, sulphides and phenols are susceptible to electron transfer oxidation to give N-oxides, hydroxylamine, sulfones and sulfoxide. The functional group with labile hydrogen like benzylic carbon, allylic carbon, and tertiary carbon or α-positions with respect to hetero atom is susceptible to oxidation to form hydro peroxides, hydroxide or ketone.

3. Thermal condition

Thermal degradation (e.g., dry heat and wet heat) should be carried out at more strenuous conditions than recommended ICH Q1A accelerated testing conditions. Samples of solid-state drug sub-stances and drug products should be exposed to dry and wet heat, while liquid drug products should be exposed to dry heat. Studies may be conducted at higher temperatures for a shorter period. Effect of temperature on thermal degradation of a substance is studied through the Arrhenius equation:

K ¼ Ae _Ea=RT

Where, k - is specific reaction rate, A is frequency factor,

Ea - is energy of activation,

R -is gas constant (1.987 cal/deg mole) and

T - is absolute temperature.

Thermal degradation study is carried out at 40°–80°C.

4. Photolytic condition

The photo stability testing of drug substances must be evaluated to demonstrate that a light exposure does not result in unacceptable change. Photo stability studies are performed to generate primary degradant of drug substance by exposure to UV or fluorescent conditions. The most commonly accepted wavelength of light is in the range of 300– 800 nm to cause the photolytic degradation. Light stress conditions can induce photo oxidation by free radical mechanism. Functional groups like carbonyls, nitro aromatic, N-oxide, alkenes, aryl chlorides, weak C–H and O–H bonds, sulphides and polyenes are likely to introduce drug photosensitivity.

Limits for degradation

Degradation of drug substance between 5% and 20% has been accepted as reasonable for validation of chromatographic assays. Some pharmaceutical scientist think 10% degradation is optimal for use in analytical validation for small pharmaceutical molecules for which acceptable stability limits of 90% of label claim are common.

Table No. 1.2: Thresholds for degradation products in drug products

Maximum daily dose[a]	**Reporting threshold [b,c]**
≤1 g	0.1%
>1 g	0.05%
Maximum daily dose[a]	**Identification threshold [b,c]**
<1 mg	1.0% or 5 µg TDI, whichever is lower
1 mg–10 mg	0.5% or 20 µg TDI, whichever is lower
>10 mg–2 g	0.2% or 2 mg TDI, whichever is
>2 g	

	lower 0.10%
Maximum daily dose[a]	**Qualification threshold [b,c]**
<10 mg 10 mg–100 mg >100 mg–2 g >2 g	1.0% or 50 µg TDI, whichever is lower 0.5% or 200 µg TDI, whichever is lower 0. 2% or 3 mg TDI, whichever is lower 0.15%

a. The amount of drug substance administered per day.

b. Thresholds for degradation products are expressed either as a percentage of the drug substance or as total daily intake (TDI) of the degradation product. Lower thresholds can be appropriate if the degradation product is unusually toxic.

c. Higher thresholds should be scientifically justified.

Impurities can be analyzed by following instruments

1. Ultra Violet spectroscopy
2. IR spectroscopy
3. NMR spectroscopy
4. Mass spectroscopy
5. Gas spectroscopy
6. HPLC

Separation Method

Capillary Electrophoresis (CE), Gas Chromatography (GC), Supercritical Fluid Chromatography (SFC), Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC), High Performance Liquid Chromatography (HPLC) is regularly being used for separation of impurities and degradation products.

Isolation Method

A list of methods that can be used for isolation of impurities is solid-phase extraction methods, Liquid-liquid extraction methods, accelerated solvent extraction methods, supercritical fluid Extraction, column chromatography, flash chromatography, capillary electrophoresis (CE), gas Chromatography (GC), thin layer chromatography (TLC), high performance thin layer Chromatography (HPTLC), high performance liquid chromatography (HPLC), supercritical fluid Chromatography (SFC).

Characterization Method

Highly sophisticated instrumentation, such as GC-MS or LC-MS are inevitable tools used in the Identification of minor components (drugs, impurities, degradation products, metabolites) in various matrices. After this identification of minor components and then their characterization can be done using NMR and MS.

High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is the most widely used analytical technique (Fig. 1.1) for the separation of the compounds from sample solution. HPLC uses a liquid mobile phase for the separation of components of a sample solution. These compounds are first dissolved in a solvent, and then forced under high pressure to flow through a chromatographic column. In the column, the mixture is resolved into its components. The amount of resolution is important, which dependents upon the extent of interaction between the solute components and the stationary phase. As a result, HPLC acquires a high degree of versatility which is not found in other chromatographic systems. HPLC has the ability to easily separate the variety of chemical mixtures. In general, highly polar materials are best separated with the use of partition chromatography, while very non polar are separated using adsorption chromatography.

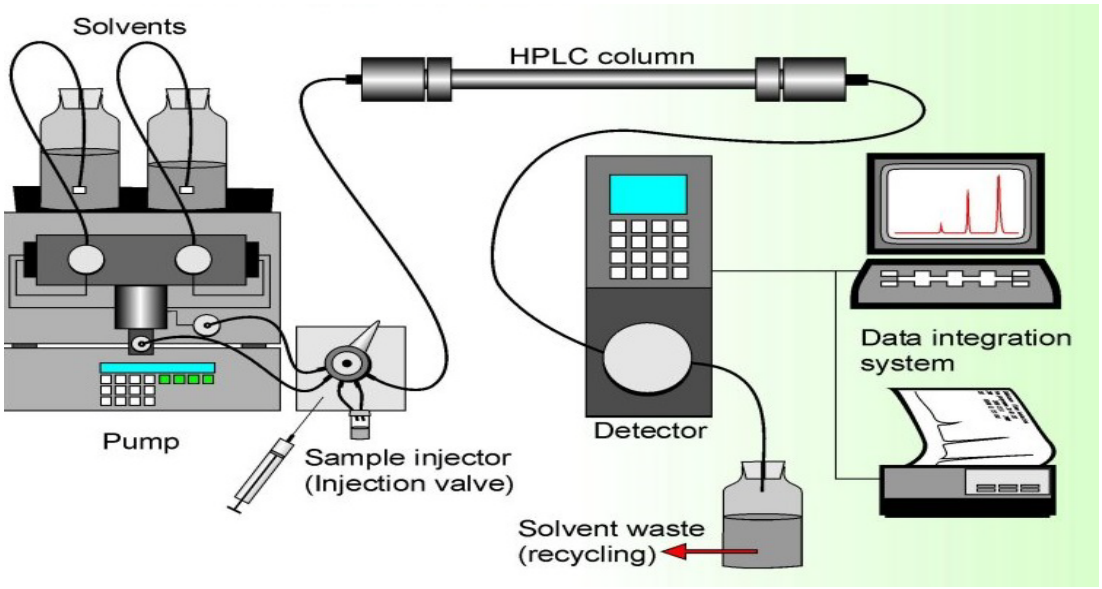

Fig. 1.1: Instrumentation of HPLC

High performance thin layer chromatography (HPTLC) is an invaluable quality assessment technique used for evaluation of botanical materials. It allows the analysis of a broad number of compounds both efficiently and cost effectively, as numbers of samples can be run in a single analysis which effectively reduces the analytical time. In HPTLC, the same analysis can be done using different wavelengths of light which provides a more complete profile of the plant materials than is typically observed with more specific types of analyses.

HPTLC is an analytical technique based on TLC, which has more enhanced intentions to increase the resolution of the compounds to be separated and to allow quantitative analysis of these compounds. Some of the enhancements such as the use of higher quality TLC plates with the fine particle sizes in stationary phase which allow better resolution. The separation can be further enhanced by the repeated development of the plate, using a multiple development device. As a consequence, HPTLC offers better resolution and lower Limit of Detection (LODs).

The use of HPTLC is well appreciated and accepted all over the world. Many methods are being developed to standardize the assay methods. HPTLC remains one step ahead when compared with other tools of chromatography.

Steps involved in HPTLC analysis

1. Selection of chromatographic layer

2. Preparation of sample and standard
3. Layer pre-washing
4. Layer pre-conditioning
5. Application of sample and standard
6. Chromatographic development
7. Detection of spots
8. Scanning
9. Documentation of chromatic plate

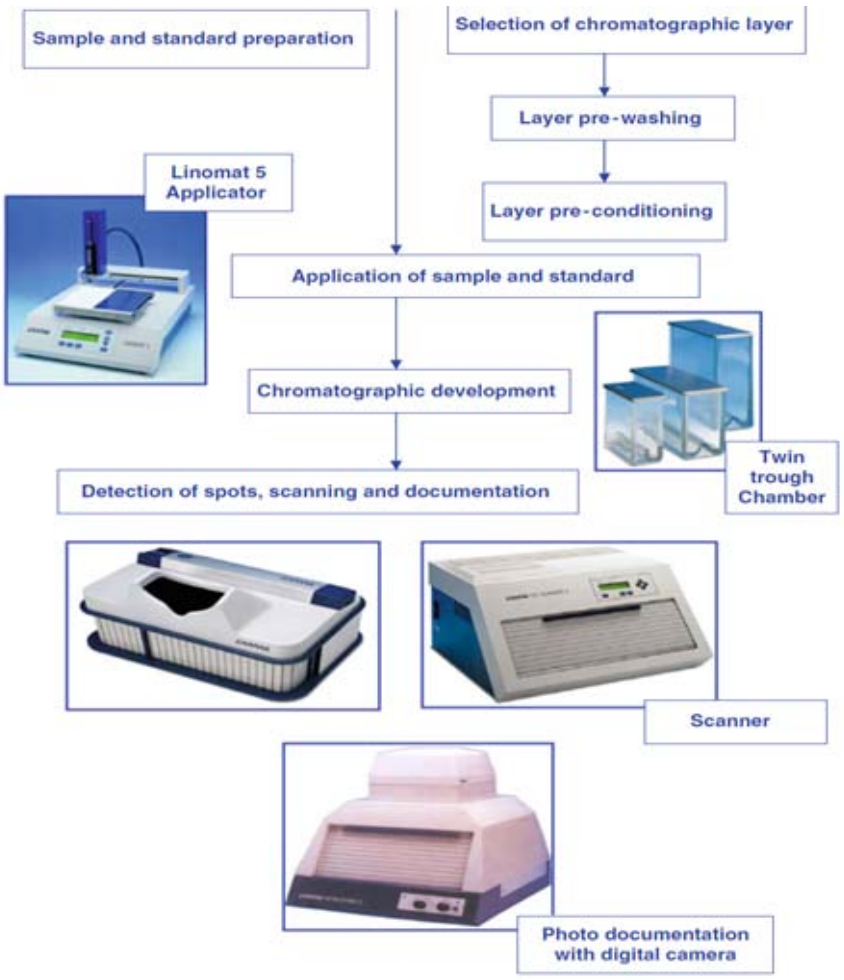

Fig. 1.2: Schematic of HPTLC instrument

Characterization methods

Highly sophisticated instrumentation, such as MS attached to a GC or HPLC, are inevitable tools in the identification of minor components (drugs, impurities, degradation products, metabolites) in various matrices. For characterization of impurities, different techniques are used; which are as follows:

MS It has an increasingly significant impact on the pharmaceutical development process over the past several decades. Advances in the design and efficiency of the interfaces, that directly connect separation techniques with Mass Spectrometers have afforded new opportunities for monitoring, characterizing, and quantification of drug-related substances in active pharmaceutical ingredients and pharmaceutical formulations. [15]

NMR The ability of NMR to provide information regarding the specific bonding structure and stereochemistry of molecules of pharmaceutical interest has made it a powerful analytical instrument for structural elucidation. The ability of NMR- based diffusion coefficient determination to distinguish between monomeric and dimeric substances was validated using a standard mixture of authentic materials containing both monomers and dimers. Unfortunately, NMR has traditionally been used as a less sensitive method compared to other analytical techniques. [16]

EVALUATION OF DEGRADANT

In general, values anywhere between 5% to 20% degradation of the drug substance have been considered as reasonable and acceptable for validation of chromatographic assays. However, for small pharmaceutical molecules for which acceptable stability limits of 90% of label claim is common, pharmaceutical scientists have agreed that approximately 10% degradation is optimal for use in analytical validation. In the event that the experimental conditions generate little or no degradants due to the exceptional stability of the molecule, an evaluation should be made to verify if the drug substance has been exposed to energy in excess of the energy provided by accelerated storage (i.e., 40°C for 6 months). If the answer is yes, then the experiment can be stopped and a note of the stability of the drug substance can be made. Unduly overstressing the drug substance may produce aberrant results.

Stress degradation study by UV-Visible Spectrophotometer[16,17]

- **Preparation of stock solution**

To prepared stock solution of Cefdinir weighed accurately 10 mg of Cefdinir bulk drug in 100 ml volumetric flask and dissolved in 80 ml of methanol solution and volume was making up to 100 ml by methanol which gives conc. of 100 µg/ml solutions.

- **Selection of analytical wavelength**

From the above stock solution 2 ml was transferred into 10 ml of volumetric flask and volume was made up to 10 ml by using methanol to give the solution of conc. of 20 ppm. Cefdinir solution of 20 ppm was scanned under UV-Vis spectrophotometer in the range 200-400 nm against methanol as blank and λ max was obtained at 287 nm.

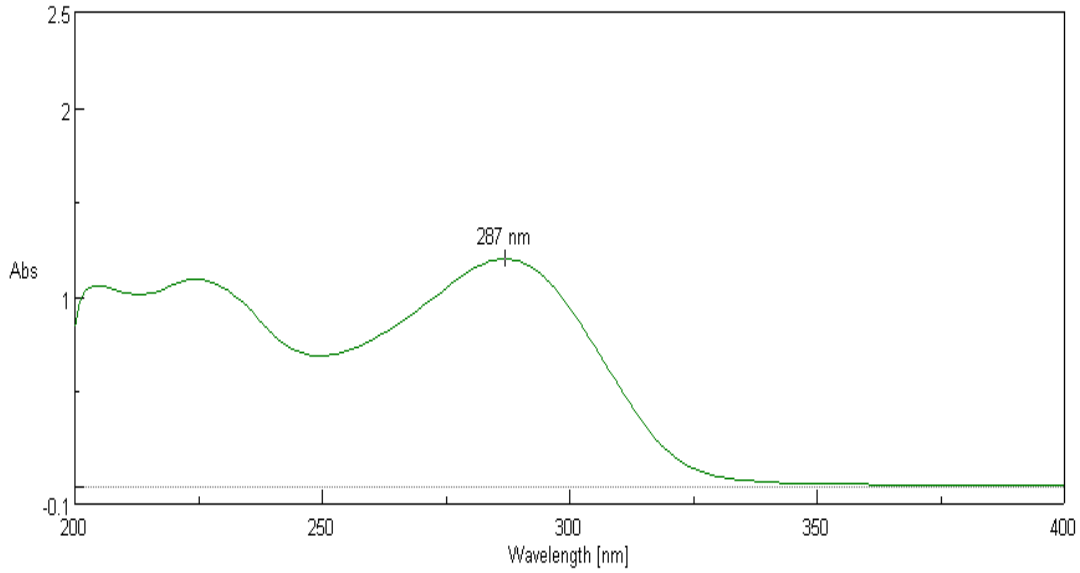

Fig. 7.1: UV spectrum of Bulk drug of Cefdinir

- **Stability of drugs in the selected Solvents:**

The stability of drug in the selected solvent was checked by measuring the absorbance of the drug solutions at various time intervals. The absorbance was measured after every 30 min for 6 hrs. The drug was found to be stable.

Table No. 7.1: Stability of 20 ppm solution of Cefdinir

Sr. no.	Concentration (ppm)	Time (min)	Absorbance (at 287 nm)
1.	20	0	1.021
2.	20	30	1.001
3.	20	60	1.021

4.	20	90	1.023
5.	20	120	1.022
6.	20	150	1.001
7.	20	180	1.001
8.	20	210	1.012
9.	20	240	1.021
10.	20	270	1.023
11.	20	300	1.021
12.	20	330	1.012
13.	20	360	1.011

(A) Method validation

Validation is process of establishing documented evidence, which provides a high degree of assurance that specific activity will consistently produce desired result or product meeting its predetermined specification and quality characteristics.

1. **Linearity**

Preparation of calibration curve

From the stock solution (100 µg/ml) prepared 0.4 – 2.4 ml was diluted by using Methanol up to 10 ml to produce 4- 24 µg/ml solutions respectively. The absorbance was taken on 287 nm and standard calibration curve was plotted as Absorbance Vs Concentration. This straight line obeyed linearity in the concentration range of 4-24 µg/ml. The correlation was found to be 0.998.

Table No. 7.2: Linearity of Cefdinir in working standard

Sr. no.	Concentration (ppm)	Absorbance
1	4	0.217
2	8	0.432
3	12	0.631
4	16	0.840
5	20	1.012
6	24	1.195

*Average of six determination.

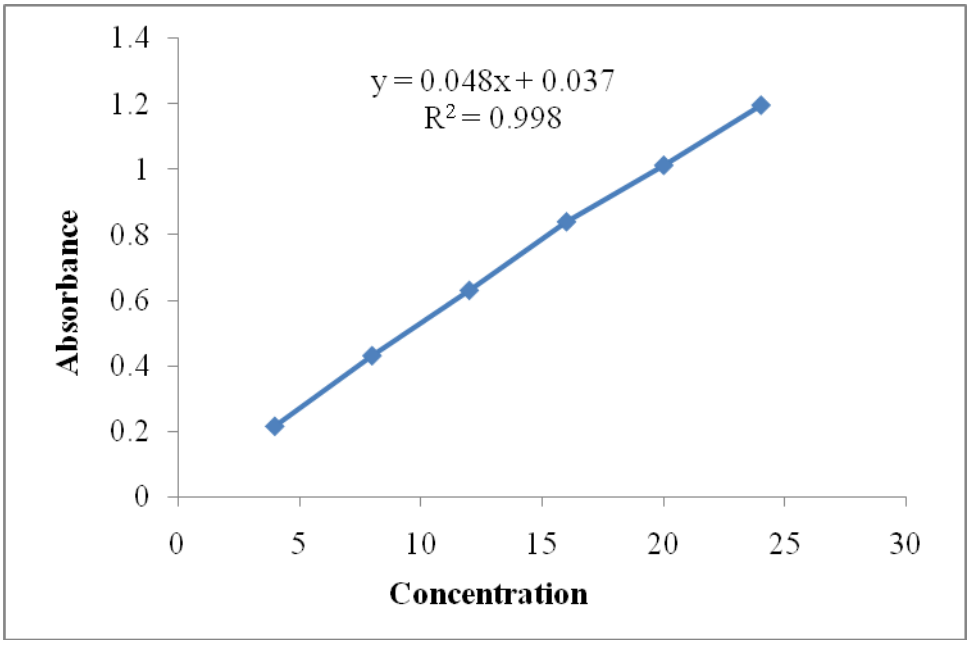

Fig. 7.2: Standard calibration curve of Cefdinir

Table No. 7.3: Optical Characteristics of Cefdinir

Parameters	Result
Beer's law limit (µg/ml)	4 - 24 µg/ml
Correlation coefficient	0.998
Regression equation (Y*)	0.048x + 0.037
Slope (a)	0.048 x
Intercept (b)	0.037

2. Accuracy

In test concentration at levels of 80%, 100%, and 120% solutions were prepared in triplicate using Cefdinir working standard as per the method and absorbance was taken of each solution in triplicate. The recovery result showed that the proposed method has an acceptable level of accuracy for level for Cefdinir which is from 80% - 120% of test concentration is from 93.70% - 101.82%.

In pure drug proportion was 10 µg/ml i.e. 1 ml; consider as 100% so calculate the 80% and 120% level of recovery and calculated how much standard (pure drug) solution was added into the tablet solution.

So; for 80%:	for 100%:	for 120%:
100% = 1 ml	100% = 1 ml	100% = 1 ml
80% = X	100% = X	120% = X
X = 0.80 ml	X = 1 ml	X = 1.2 ml

Then added the standard (bulk drug) solution + tablet solution

10 µg/ml i.e. 1 ml tablet solution reading

80% = 1 ml from tablet stock solution + 0.80 ml from bulk drug stock solution = 18 µg/ml

100% = 1 ml from tablet stock solution + 1 ml from bulk drug stock solution = 20 µg/ml recovered

120% = 1 ml from tablet stock solution + 1.2 ml from bulk drug stock solution = 22 µg/ml recovered

Calculation was performed with the using following formula:

$$\text{Conc. found } (\mu g/ml) = \frac{\text{Absorbance at recovered level x standard concentration of tablet solution } (\mu g/ml)}{\text{Absorbance of tablet solution}}$$

$$\% \text{ Recovery} = \frac{\text{Concentration of drug found } (\mu g/ml) \times 100}{\text{Concentration of standard solution at 100\%}}$$

Table No. 7.4: Accuracy study of Cefdinir bulk drug

No. of Preparation	Concentration (µg/ml)		% Recovery	Mean
	Formulation	Pure drug		
S_1:80%	10	8	94.23	93.70%
S_2:80%	10	8	91.34	
S_3:80%	10	8	95.55	
S_1:100%	10	10	92.18	94.68%
S_2:100%	10	10	95.20	
S_3:100%	10	10	96.66	
S_1:120%	10	12	102.46	101.82%
S_2:120%	10	12	101.32	
S_3:120%	10	12	101.82	

3. Precision

Precision of the method was demonstrated by intraday and interday variation studies. In intraday variation study three different solutions of three different concentrations were analyzed in a day i.e. from morning, afternoon and evening. In the interday variation studies, solution of three different concentration were analyzed three times for the three consecutive days and the absorbance result mean, standard deviation (S) and % RSD was calculated .

$$s = \sqrt{\frac{\sum_{i=1}^{n}(X_i - \overline{X})^2}{n-1}}$$

Where,

X = individual value

X = arithmetic mean

n = number of samples

Or Coefficient of variation (C.O.V)

% Relative standard deviation (%R.S.D.) = SD/X x 100

Where,

SD= Standard Deviation

X= Mean

Table No. 7.5: Intra-day precision studies for Cefdinir

Conc. (µg/ml)	Absorbance (nm)			Mean	SD	% RSD
	Trial 1	Trial 2	Trial 3			
4	0.217	0.214	0.207	0.2126	±0.00419	1.97
8	0.432	0.422	0.431	0.4283	±0.00550	1.28
12	0.631	0.612	0.633	0.6253	±0.01159	1.85
Average Of % RSD=1.7%						

Table No. 7.6: Inter-day precision studies for Cefdinir

Conc. (µg/ml)	Absorbance (nm)			Mean	SD	% RSD
	Day 1	Day 2	Day 3			
4	0.199	0.201	0.195	0.198	±0.00305	1.54
8	0.423	0.430	0.422	0.425	±0.00435	1.025
12	0.671	0.673	0.677	0.673	±0.00305	0.452
Average OF % RSD= 1.005%						

4. Robustness

Robustness of the method was determined by carrying out the analysis under different temperature condition i.e. at room temperature and at 18°C. The respective absorbances of 20 µg/ml were noted and the result was indicated as %RSD.

Table No. 7.7: Robustness for Cefdinir bulk drug

Sr. no	Concentration (ppm)	Absorbance	
		Room temperature	18^0C
1	20	0.988	0.988
2	20	0.981	0.983
3	20	0.985	0.986
4	20	0.986	0.987
5	20	0.990	0.991
6	20	0.989	0.989
Mean		0.9865	0.8466
SD		0.00327	0.002733
%RSD		0.331	0.322
Average% RSD=0.3265%			

5. Ruggedness

Ruggedness of the method was determined by carrying out the analysis by different analyst and the respective absorbance of 20 µg/ml was noted. The result was indicated as %RSD.

$$s = \sqrt{\frac{\sum_{i=1}^{n}(X_i - \overline{X})^2}{n-1}}$$

Where,

X = individual value

X = arithmetic mean

n = number of samples

% Relative standard deviation (%R.S.D.) = S/X x 100

Where,

S= Standard Deviation

X= Mean

Table No. 7.8: Ruggedness for Cefdinir bulk drug

Sr. no	**Conc. (ppm)**	**Absorbance**		
		Analyst 1	**Analyst 2**	**Analyst 3**
1	20	0.999	0.999	0.999
2	20	0.998	0.998	0.997
3	20	0.998	0.998	0.998
4	20	0.997	0.997	0.996
5	20	0.996	1.011	1.011
6	20	1.011	0.999	0.999
Mean		0.9998	1.0003	1.000
SD		0.005565	0.005279	0.005514
% RSD		0.5566	0.5277	0.5514
Average% RSD= 0.5452%				

6. Limit of detection (LOD)

The limit of detection (LOD) was separately determined based on the standard deviation of response of the calibration curve. The standard deviation of the y intercept and slope of the calibration curve were used.

LOD is calculated from the formula: -

$$\mathbf{LOD = 3.3 \times S.D / S}$$

Where,

LOD = limit of detection

3.3 = Standard Factor

S.D = standard deviation of response for the lowest conc. in the range

S = slope of the calibration curve.

LOD = 3.3 X 0.000577 / 0.048

LOD was found to be 0.039 µg/ml

7. Limit of quantification (LOQ)

The LOQ is the concentration that can be quantification reliably with a specified level of accuracy and precision. The LOQ was calculated using the formula involving standard deviation of response and slope of calibration curve .The quantitation limit (QL) may be expressed as:

$$\mathbf{LOQ = 10x\ S.D/S}$$

Where,

LOQ = Limit of quantification

10 = Standard Factor

S.D = standard deviation of response for the lowest conc. in the range

S = slope of the calibration curve.

LOQ= 10 X0.000577/ 0.048

LOQ was found to be 0.120 µg /ml.

Table No. 7.9: Summary of validation of Cefdinir bulk drug

Sr. No.	Parameter	Result
1	Linearity indicated by correlation coefficient	0.998
2	Linear regression equation	0.048x + 0.037
3	Range	4 µg/ml – 24 µg/ml
4	Intraday Precision (%RSD)	1.7%
5	Interday Precision (%RSD)	1.005%
6	Limit of Detection	0.039 µg/ml
7	Limit of Quantification	0.120 µg/ml
8	Robustness indicated by % RSD	0.3265%
9	Ruggedness indicated by % RSD	0.5452%

(B) Stress degradation studies

The International Conference on Harmonization (ICH) guidelines entitled stability testing of new drug substance and products requires that stress testing carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform impurity studies on the Cefdinir using proposed method.

1. Acidic degradation:

In six different volumetric flasks, 2 ml of stock solution of Cefdinir bulk drug was added and mixed with 3 ml of 0.1N, 0.5N, 1N, 1.5N, 2N, 2.5N hydrochloric acid in each volumetric flask respectively. The volumetric flask was kept for 3 hour at room temperature. After every 1 hour time interval, solution was neutralized and diluted with methanol in order to make up the volume up to 10 ml and the dilution was carried out to achieve the appropriate conc. (20 µg/ml). The degradation was observed in the 0.1N hydrochloric acid at time interval of 1 hour. (Fig. 7.3 and Table No. 7.10)

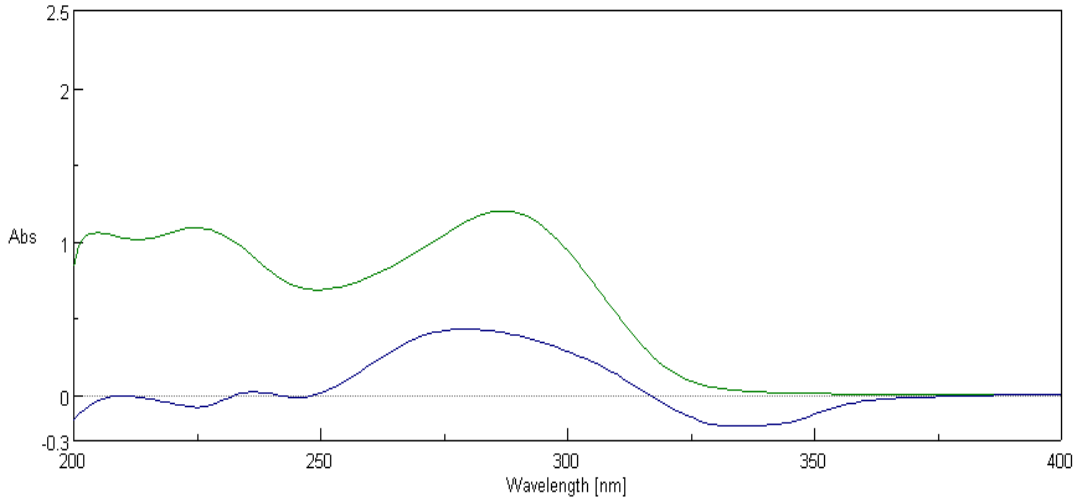

Fig. 7.3: Comparison between pure drug and acid degraded sample of Cefdinir.

- **% purity of standard drug without stress condition**

Absorbance of standard solution (20 ppm) at 287nm = 0.987

Amount of drug present in standard solution was x

From regression equation were finding out drug content of 20 ppm solution i. e.

$$Y = m x + c$$

Where, Y = Absorbance; m = Slope

x = Concentration c = Constant

$$Y= 0.048x + 0.037$$

$$0.987= 0.048x + 0.037$$

$$X= 19.79 \mu g/ml$$

The drug present in 20 ppm solution is **19.79 µg/ml**

% drug content in given solution was calculated as,

20 µg /ml corresponds to 100%, so 19.79 µg /ml corresponds to A%

$$A= 19.79 \times 100/ 20$$

$$A = 98.95\%$$

The % drug content in standard solution of 20 ppm was found to be **98.95%**

- **% purity of drug after acidic condition**

Absorbance of acid degraded sample (20 ppm) at 287 nm = 0.840

Amount of drug present in acid degraded solution was x

From regression equation were finding out drug content of degraded sample of 20 ppm solution i. e.

$$Y = m x + c$$

Where, Y = Absorbance; m = Slope

x = Concentration c = Constant

$$Y= 0.048x + 0.037$$

$$0.840= 0.048x + 0.037$$

$$X=16.72 \mu g /ml.$$

The drug present in degraded 20 ppm solution was **16.72 µg /ml.**

% Drug content in given degraded solution was calculated as,

20 µg /ml corresponds to 100%, so 16.72 µg /ml corresponds to B%

B= 16.72 x 100/20

B= 83.64%

The % drug content in degraded solution of 20 ppm was found to be **83.64%**

The difference between the percent drug content of standard solution and percent drug content of degraded solution gives the percentage of drug degraded.

98.95-83.64=15.31%

Therefore, **15.31% drug has been degraded in acidic condition.**

The difference between the amount of drug present in standard solution and the amount of drug present in degraded solution gives the amount of drug degraded

19.79-16.72=3.07 µg /ml

3.07 µg /ml of drug have been degraded in acidic condition.

2. Alkali Degradation

In six different volumetric flasks 2 ml of stock solution of Cefdinir bulk drug was added and 3 ml of 1N, 1.5N, 2N, 2.5N, 3N, 3.5N sodium hydroxide was added in each volumetric flask respectively. The volumetric flask was kept for 3 hours at room temperature. After every 1 hour time interval, solution was neutralized and diluted with methanol in order to make the volume up to 10 ml and the dilution was carried out to achieve the appropriate conc. (20 µg/ml). The degradation was observed in the 0.1N sodium hydroxide at interval of 1 hour. (Fig. 7.4 and Table No. 7.10)

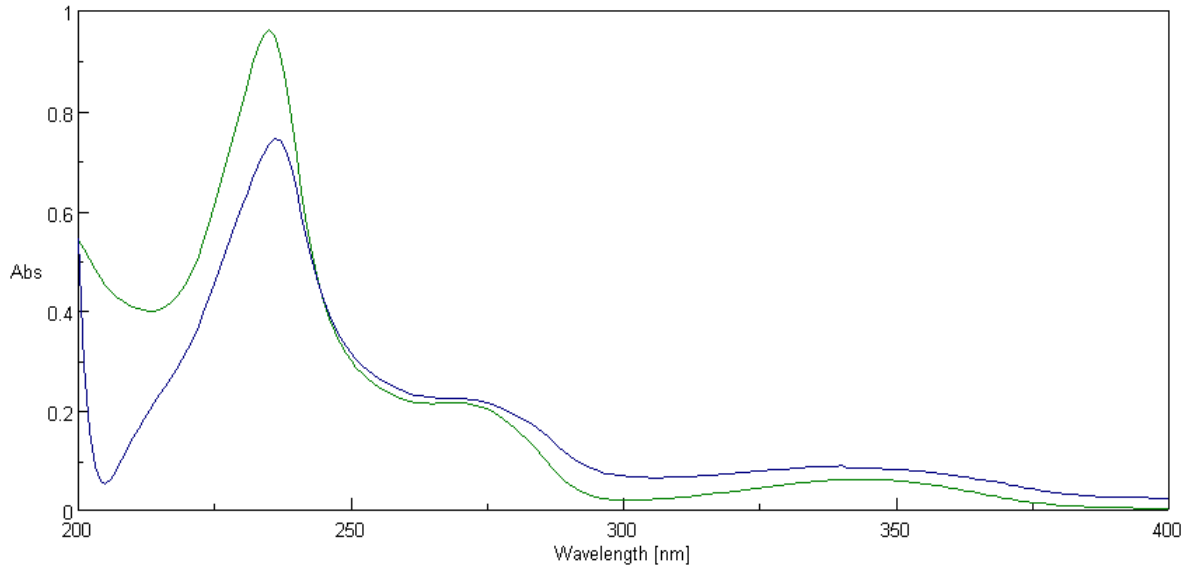

Fig. 7.4: Comparison between bulk drug and alkaline degraded sample of Cefdinir.

- **% purity of standard drug without stress condition**

The drug present in 20 ppm solution is **19.79 µg/ml**

The % drug content in standard solution of 20 ppm was found to be **98.95%**

- **% purity of degraded drug with alkaline condition**

Absorbance of alkali degraded sample (20 ppm) at 287 nm= 0.881

Amount of drug present in alkali degraded solution was x

From regression equation were finding out drug content of alkaline degraded solution of 20 ppm solution i. e.

$$Y = m x + c$$

Where, Y = Absorbance; m = Slope

x = Concentration c = Constant

$$Y= 0.048x + 0.037$$

$$0.881= 0.048x + 0.037$$

$$X= 17.58 \text{ µg/ml.}$$

The drug present in degraded 20 ppm solution was **17.58 µg/ml.**

% Drug content in given degraded solution was calculated as,

20 µg /ml corresponds to 100%, so 17.58 µg /ml corresponds to B%

B= 17.58 x 100/20

B= 87.91%

The % drug content in degraded solution of 20 ppm was found to be 87.91%

The difference between the percent drug content of standard solution and percent drug content of degraded solution gives the percentage of drug degraded.

98.95-87.91=11.04%

Therefore, **11.04 % drug has been degraded in alkaline condition**

The difference between the amount of drug present in standard solution and the amount of drug present in degraded solution gives the amount of drug degraded.

19.79-17.58=2.21 µg /ml

2.21 µg /ml of drug has been degraded in alkaline condition

3. Photolytic degradation

Sample of Cefdinir was exposed to UV light for 24 hours. 20 mg sample was dissolved in methanol and volume was made up to 10 ml. From this solution appropriate dilution (20 µg/ml) was made using methanol and taken in cuvette for the UV analysis. (Fig. 7.5 and Table No. 7.10)

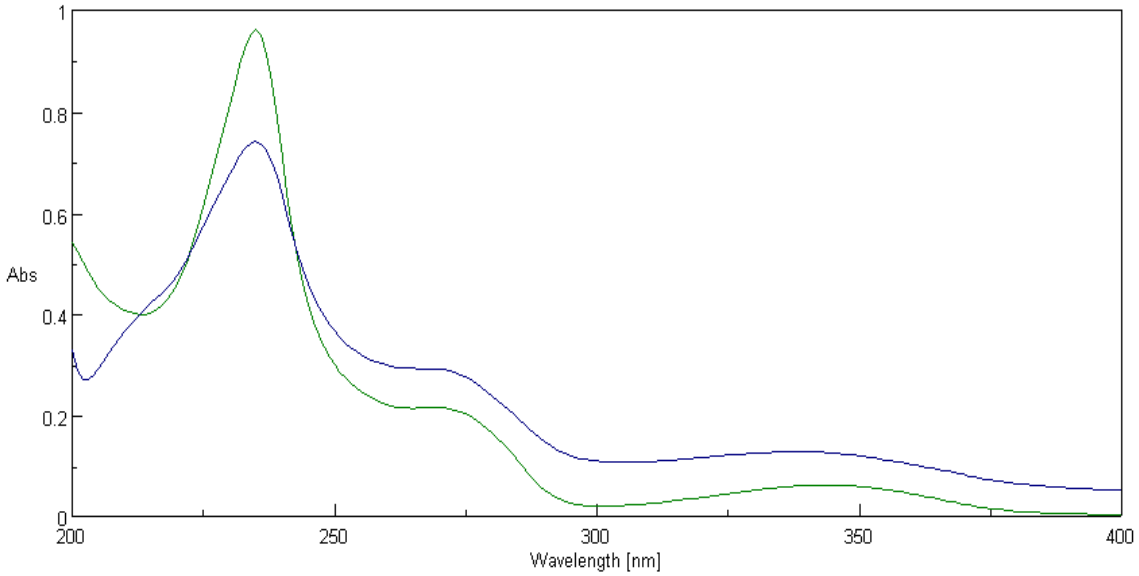

Fig. 7.5: Comparison between bulk drug and under photo degraded sample of Cefdinir

- **% purity of standard drug without stress condition**

The drug present in 20 ppm solution is **19.79 µg/ml**

The % drug content in standard solution of 20 ppm was found to be **98.95%**

- **% purity of degraded drug with photo degradation**

Absorbance of photo degraded sample (20 ppm) at 287 nm= 0.975

Amount of drug present in photo degraded solution was x

From regression equation were finding out drug content of photo degraded solution of 20 ppm solution i. e.

$$Y = m x + c$$

Where, Y = Absorbance; m = Slope

x = Concentration c = Constant

$$Y= 0.048x + 0.037$$

$$0.975= 0.048x + 0.037$$

$$X= 19.54 \text{ µg /ml.}$$

The drug present in degraded 20 ppm solution was 19.54 µg/ml.

% Drug content in given degraded solution was calculated as,

20 µg /ml corresponds to 100%, so 19.54 µg /ml. corresponds to B%

B= 19.54 x 100/20

B= 97.70%

The difference between the percent drug content of standard solution and percent drug content of degraded solution gives the percentage of drug degraded.

98.95-97.70=1.25%

Therefore, **1.25% drug has been degraded in photo degradation condition**

The difference between the amount of drug present in standard solution and the amount of drug present in degraded solution gives the amount of drug degraded.

19.79-19.54=0.25 µg/ml

0.25 µg/ml of drug have been degraded in photo degradation condition.

4. Thermal degradation

Cefdinir sample was taken in a petriplate and exposed to a temperature of 70°C for 48 hours in an oven. Remove the sample from oven and kept it at room temperature for 1 hour, After 1 hour, 20 mg of the sample was diluted with methanol in order to make the volume up to 10 ml. From this solution, dilutions were carried out to achieve the appropriate conc. (20 µg/ml) solution taken in cuvette for UV-Vis Analysis. (Fig. 7.6 and Table No. 7.10)

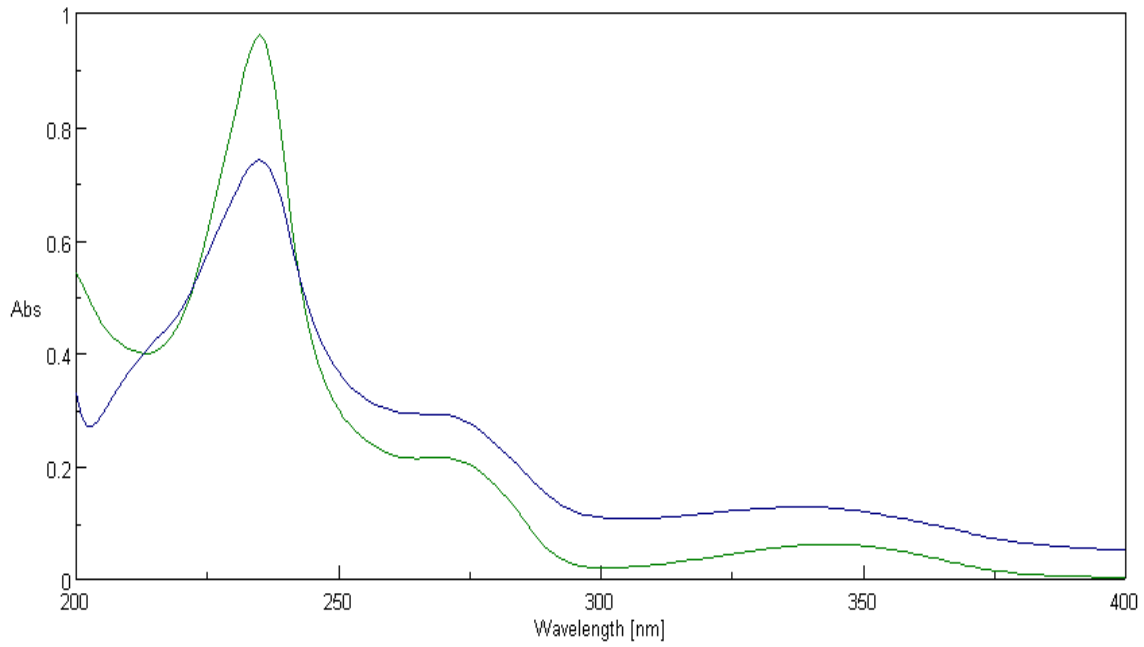

Fig. 7.6: Comparison between bulk and thermal degraded sample of Cefdinir.

- **% purity of standard drug without stress condition**

The drug present in 20 ppm solution is **19.79 µg/ml**

The % drug content in standard solution of 20 ppm was found to be **98.95%**

- **% purity of degraded drug with thermal condition**

Absorbance of thermal degraded sample (20 ppm) at 287 nm= 0.962

Amount of drug present in thermal degraded solution was x

From regression equation were finding out drug content of oxidative degraded solution of 20 ppm solution i. e.

$$Y = m x + c$$

Where, Y = Absorbance; m = Slope

x = Concentration c = Constant

$$Y= 0.048x + 0.037$$

$$0.962= 0.048x + 0.037$$

$$X= 19.27 \text{ µg/ml.}$$

The drug present in degraded 20 ppm solution was 19.27 µg /ml.

% Drug content in given degraded solution was calculated as,

20 µg /ml corresponds to 100%, so 19.27 µg /ml. corresponds to B%

B= 19.27 x 100/20

B=96.35%

The difference between the percent drug content of standard solution and percent drug content of degraded solution gives the percentage of drug degraded.

98.95-96.35=2.6%

Therefore, **2.6 % drug has been degraded in thermal condition**

The difference between the amount of drug present in standard solution and the amount of drug present in degraded solution gives the amount of drug degraded.

19.79-19.27=0.52 µg /ml

0.52 µg /ml of drug has been degraded in thermal condition

5. Oxidative degradation

In six different volumetric flasks 2 ml of stock solution of Cefdinir bulk drug was added and 3 ml of 1%, 3%, 6%, 20%, 30% v/v of hydrogen peroxide was added in each volumetric flask respectively. The volumetric flask was kept for 3 hours. After every 1 hour time interval dilution was carried out to achieve the appropriate conc. (20 µg/ml). The degradation was observed in the 6% v/v of hydrogen peroxide. (Fig. 7.7 and Table No. 7.10)

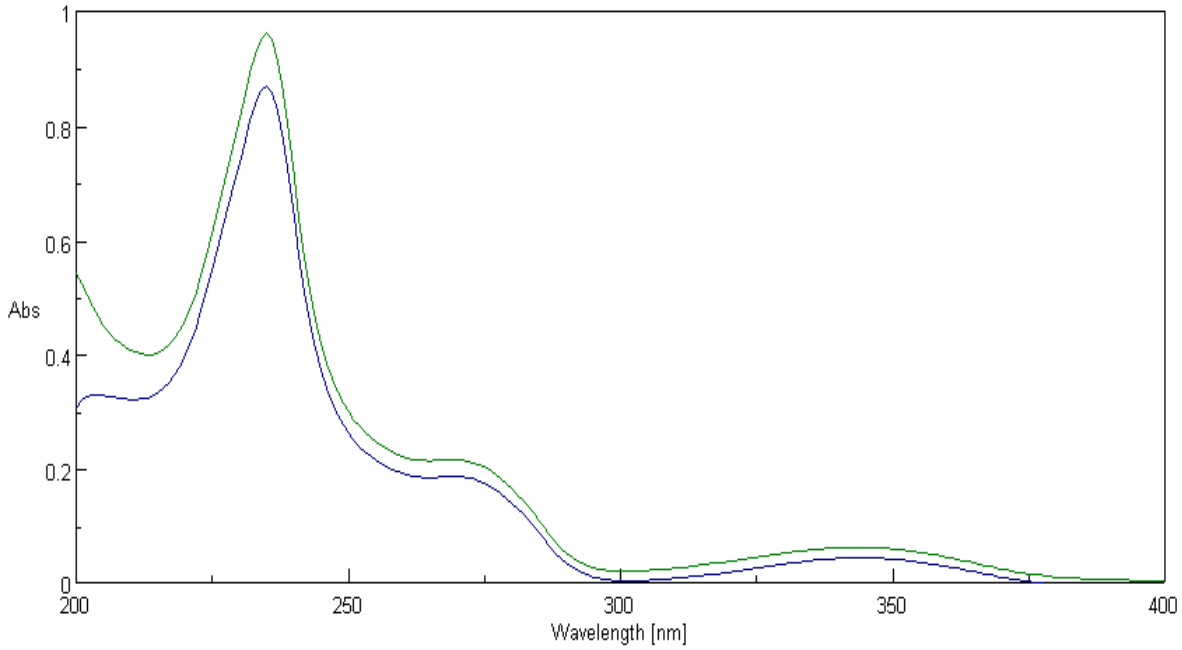

Fig. 7.7: Comparison between bulk drug and oxidative degraded sample of Cefdinir.

- **% purity of standard drug without stress condition**

The drug present in 20 ppm solution is **19.79 µg/ml**

The % drug content in standard solution of 20 ppm was found to be **19.79%**

- **% purity of degraded drug with oxidative condition**

Absorbance of oxidative degraded sample (20 ppm) at 287 nm= 0.969

Amount of drug present in oxidative degraded solution is x

From regression equation we were finding out drug content of oxidative degraded solution of 20 ppm solution i. e.

$$Y = m x + c$$

Where, Y = Absorbance; m = Slope

x = Concentration c = Constant

$$Y= 0.048x + 0.037$$

$$0.969= 0.048x + 0.037$$

$$X=19.41 \text{ µg /ml.}$$

The drug present in degraded 20 ppm solution was 19.41 µg /ml.

% Drug content in given degraded solution was calculated as,

20 µg /ml corresponds to 100%, so 19.41 µg /ml corresponds to B%

B=19.41x 100/20

B= 97.08%

The difference between the percent drug content of standard solution and percent drug content of degraded solution gives the percentage of drug degraded.

98.95-97.08=1.87%

Therefore, **1.87% drug has been degraded in oxidative condition.**

The difference between the amount of drug present in standard solution and the amount of drug present in degraded solution gives the amount of drug degraded.

19.79-19.41=0.38 µg /ml

0.38 µg /ml of drug have been degraded in oxidative condition.

Table No. 7.10: Summary of result of stress degradation studies

Stress condition	Time	Observation	Concentration of Cefdinir degraded (µg/ml)	% Degradation
Acidic Degradation	Reflux 1 hours	λ max shifted	3.07	15.31
Alkali Degradation	Reflux 1 hours	λ max shifted	2.21	11.04
Photo degradation	24 hours	No λ max shifted	0.25	1.25
Thermal degradation	48 hours	No λ max shifted	0.52	2.6
Oxidative degradation	RT 1 hours	No λ max shifted	0.38	1.87

*RT- Room Temperature

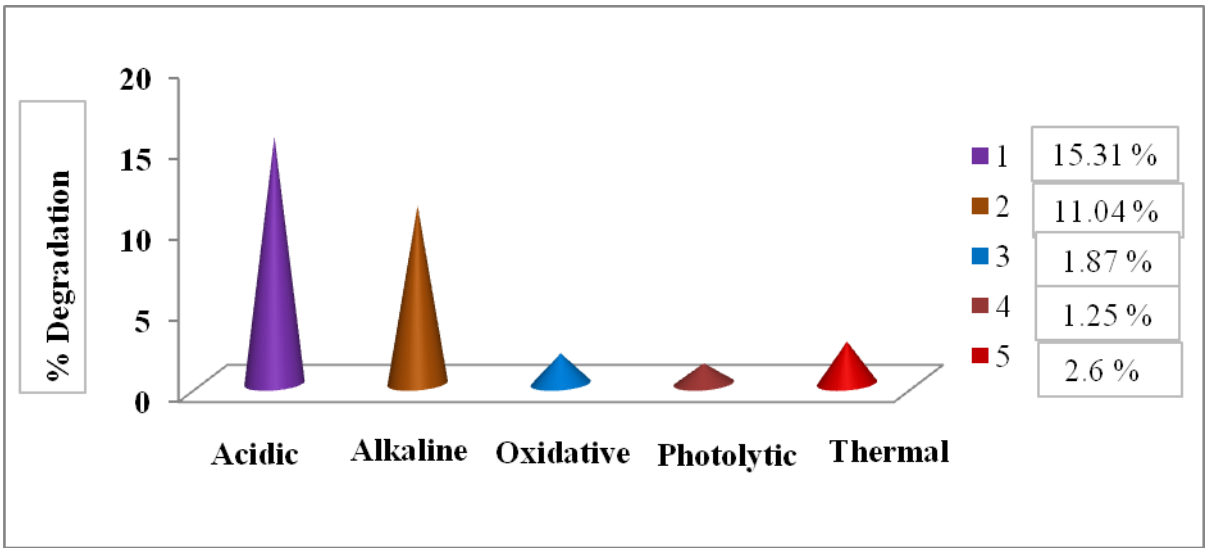

Fig. 7.8: Statistical representation of all degradation conditions

Stress degradation study by HPLC[18,19]

From the standard stock solution (100 µg/ml) further dilutions were done using Phosphate buffer (pH-6.8) : Acetonitrile (60:40 v/v) and scanned over the range of 200 - 400 nm and the spectra was obtained. It was observed that Cefdinir showed considerable absorbance at 287 nm.

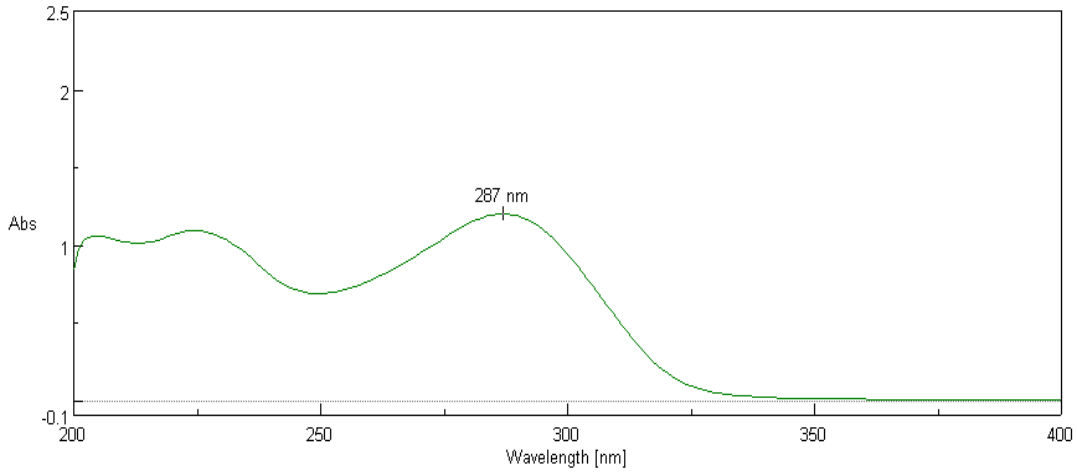

Fig. 8.1: UV spectrum of Cefdinir in Phosphate buffer (pH-6.8): Acetonitrile (60:40 v/v)

- **Selection of Mobile Phase and Chromatographic Conditions**

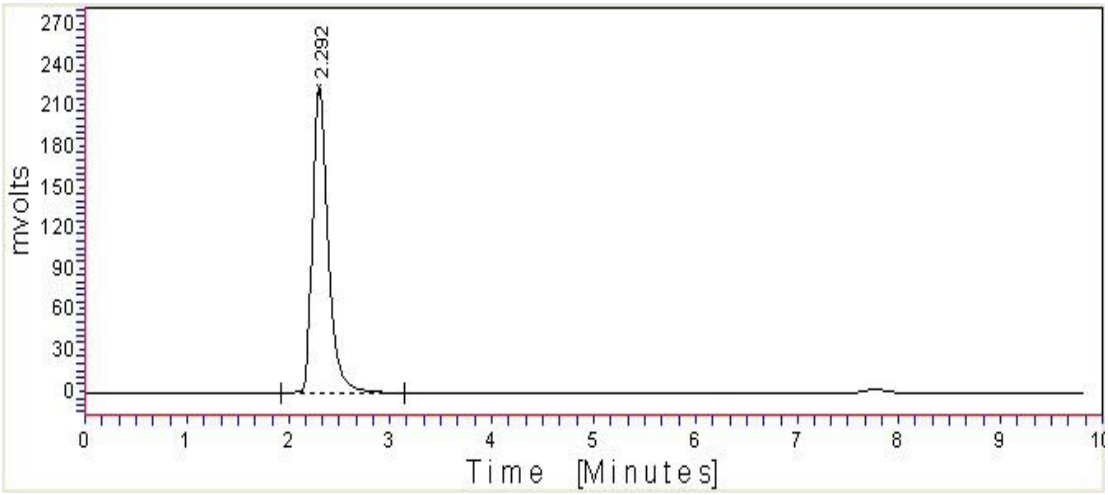

Fig. 8.2: Chromatogram of Cefdinir with mobile phase Phosphate buffer (pH-6.8): Acetonitrile (60:40 v/v)

Preparation of Standard stock solution

To prepared a stock solution of Cefdinir weighed accurately 10 mg of Cefdinir bulk drug was transferred in 100 ml volumetric flask. Drug was dissolve in Phosphate buffer (pH-6.8) and volume was made up to 100 ml with same solvent. So as to get the conc. of 100 µg/ml. Pipette out 2 ml of standard stock solution of cefdinir in 10 ml of volumetric flask was then diluted in 10 ml Phosphate buffer (pH-6.8) : Acetonitrile (60:40 v/v) to get working standard solution 20 µg/ml.

- **Preparation of Mobile Phase:**

Prepared a mobile phase by mixing Phosphate buffer (pH-6.8): Acetonitrile (60:40 v/v) filtered through 0.45µ membrane filter paper and then sonicated on ultrasonic water bath for 15 min.

- **Chromatogram of Cefdinir**

The column was saturated with the mobile phase (indicated by constant back pressure at desired flow rate). Standard solution of Cefdinir was injected to get the chromatogram. The retention time for Cefdinir was found to be at 2.29 min.

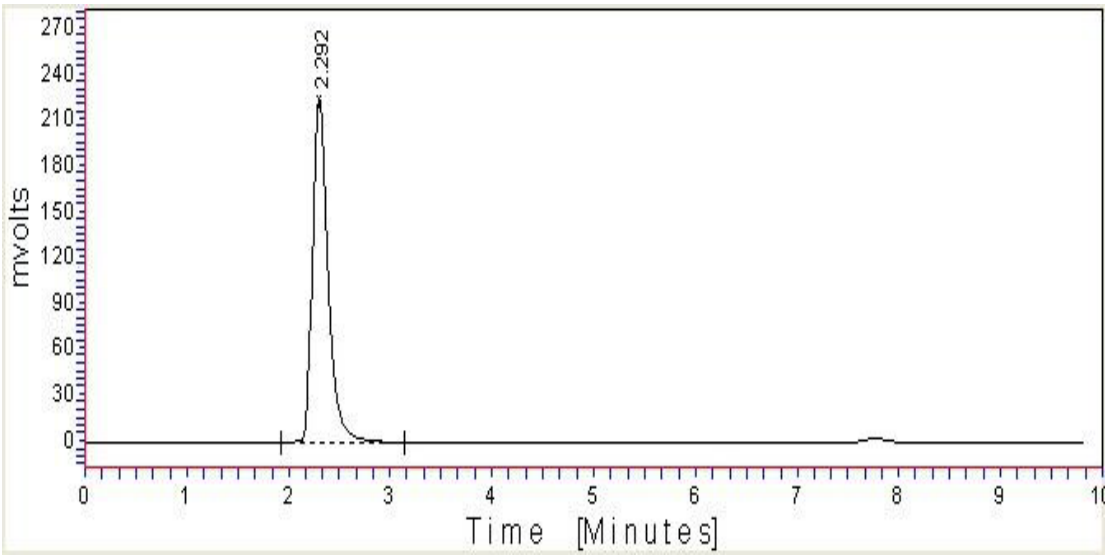

Fig. 8.3: Chromatogram of Cefdinir (20 µg/ml, RT = 2.292) with mobile phase phosphate buffer (pH- 6.8): Acetonitrile (60: 40)

- **Summary of chromatographic conditions selected**

1. Column : Grace smart C_{18} (250 x 4.6 mm i.d)

2. Mobile phase : phosphate buffer (pH- 6.8) : Acetonitrile (60 : 40)
3. Wavelength : 287 nm.
4. Loop : 20 µl.
5. Flow rate : 1.0 ml/min.
6. Temperature: Ambient.

[A] Method validation

1. Linearity:

From the stock solution (100 µg/ml) prepared 0.4 – 2.4 ml was diluted by using methanol up to 10 ml to produce 4- 24 µg/ml solutions respectively. The peak area was calculated on 287 nm and standard calibration curve was plotted as Peak area Vs Concentration. This straight line obeyed linearity in the concentration range of 4-20 µg/ml. The correlation was found to be 0.997. Shown in table no. 8.1 and graph no. 8.4.

Table No. 8.1: Linearity studies of Cefdinir

Sr. no.	Concentration	Peak area
1	4 µg/ml	11454.11
2	8 µg/ml	23857.33
3	12 µg/ml	31231.13
4	16 µg/ml	42672.21
5	20 µg/ml	53199.32
6	24 µg/ml	62581.28

*Average of six determination

Fig. 8.4: Standard calibration curve for Cefdinir

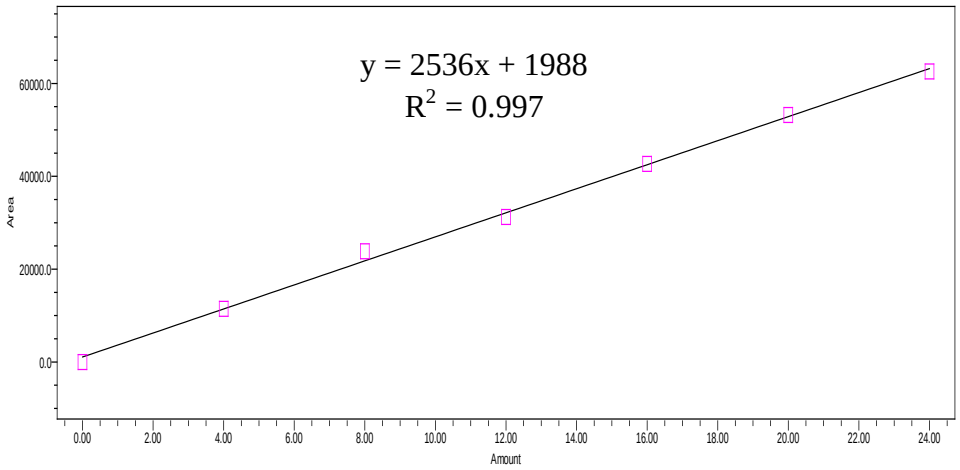

Table No. 8.2: Optical Characteristics

Beer's law limit (µg/ml)	4-24 µg/ml
Correlation coefficient	0.997
Regression equation (Y*)	2536x + 1988
Slope (a)	1988
Intercept (b)	2536x

2. Precision

The precision of the method was demonstrated by intra-day and inter-day variation studies. In the inter day studies, 3 different concentrations 4, 8 and 12 µg/ml were injected in stabilized chromatographic conditions and were analyzed in triplicate. The percentage RSD was calculated. The result obtained for intraday variations are shown in table no. 8.3.

In the inter day variation studies, 4, 8 and 12 µg/ml were injected in stabilized chromatographic conditions and were analyzed. This procedure was repeated once a day for three consecutive days. The percentage RSD was calculated. The result obtained for intraday variation is shown in table no. 8.4.

$$s = \sqrt{\frac{\sum_{i=1}^{n}(X_i - \overline{X})^2}{n-1}}$$

Where,

X = individual value

X = arithmetic mean

n = number of samples

% Relative standard deviation (%R.S.D.) = SD/X x 100

Where,

SD= Standard deviation

X= Mean

Table No. 8.3: Intra-day precision studies for Cefdinir bulk drug

Conc. (µg/ml)	Peak area			Mean	SD	% RSD
	Trial 1	Trial 2	Trial 3			
4	11458.10	11584.14	11668.18	11570.14	105.737	0.9138
8	23888.35	23869.31	23871.33	23876.33	10.4585	0.0438
12	31238.13	31309.17	31286.20	31277.83	36.2515	0.1159
Average of %RSD=0.3578						

Table No. 8.4: Inter-day precision studies for Cefdinir bulk drug

Conc. (µg/ml)	Peak area			Mean	SD	% RSD
	Day 1	Day 2	Day 3			
4	11452.11	11485.15	11568.19	11501.81	59.80782	0.5199
8	23828.35	23832.31	23815.33	23825.33	8.883715	0.03728
12	31228.14	31311.21	31279.23	31272.86	41.89997	0.1339
Average of %RSD=0.2303						

3. Accuracy

In test concentration at levels of 80%, 100%, and 120% solutions were prepared in triplicate using Cefdinir working standard as per the method and absorbance was taken of each solution in triplicate. Basic concentration of sample chosen was 10 µg/ml of Cefdinir tablet solution to which 8, 10 and 12 µg/ml of Cefdinir drug solution was added. These solutions were injected in stabilized chromatographic conditions in triplicate to obtain the chromatograms. The drug concentrations of Cefdinir were calculated by using linearity equation.

In pure drug proportion was 10 µg/ml i.e. 1 ml; consider as 100% so calculate the 80% and 120% level of recovery and calculated how much standard (pure drug) solution was added into the tablet?

So;	for 80%:	for 100%:	for 120%:
	100% = 1 ml	100% = 1 ml	100% = 1 ml
	80% = X	100% = X	120% = X
	X = 0.8 ml	X = 1 ml	X = 1.2 ml

Then added the standard (pure drug) solution + tablet solution

10 µg/ml i.e. 1 ml tablet solution reading

80% = 1 ml from tablet stock solution+0.8 ml from bulk drug stock

Solution = 18 µg/ml

100% = 1 ml from tablet stock solution + 1 ml from bulk drug stock solution = 20 µg/ml

120% = 1ml from tablet stock solution+ 1.2 ml from bulk drug stock solution = 22 µg/ml

Calculation was performed with the using following formula:

$$\text{Conc. Found (µg/ml)) } = \frac{\text{Peak area at recovered level x standard conc. of tablet solution (µg/ml)}}{\text{Peak area at tablet solution in µg/ml}}$$

$$\text{\% Recovery } = \frac{\text{Conc. of drug found (µg/ml) x 100}}{\text{Concentration of standard solution at 100\%}}$$

Determine the accepted concentration corresponds to peak area by calibration curve equation, and from that calculate the % recovery.

Table No. 8.5: Recovery Studies of Cefdinir

Level	Conc. (µg/ml) Std Sample	Area	Mean	Recovered Conc. (µg/ml)	% Recovery
80%	10 + 08	40335.11 41328.21 39343.15	40335.49	15.12	84.00%
100%	10 + 10	54830.41 53779.39 53825.42	541450.7	20.56	102.83%
120%	10 + 12	61855.36 59763.40 60849.32	60822.69	23.19	105.45%

4. Limit of detection (LOD)

LOD is calculated from the formula: -

LOD = 3.3 x SD/ S

Where,

LOD= Limit of detection

SD = standard deviation

3.3= Standard factor

S = Slope of the calibration curve.

LOD =3.3 x 1772.547/29357.33

LOD was found to be 0.1992 µg/ml

5. Limit of quantification (LOQ)

The Quantitation limit (QL) may be expressed as:

LOQ= 10 x SD/S

Where,

LOQ=Limit of quantification

SD = Standard deviation

10 = Standard factor

S = slope of the calibration curve.

LOQ = 10 x 1772.547/29357.33

LOQ was found to be 0.6037 µg /ml.

6. Range

Cefdinir: 4-24 µg/ml

7. **Robustness**

Robustness was performed by injecting the Cefdinir standard solution in to the HPLC by altering the flow rate, from the normal chromatographic conditions.

$$s = \sqrt{\frac{\sum_{i=1}^{n} (X_i - \overline{X})^2}{n-1}}$$

Where,

X = individual value

X = arithmetic mean

n = number of samples

% Relative standard deviation (%R.S.D.) = SD/X x 100

Where,

SD= Standard deviation

X= Mean

Table No. 8.6: Robustness Studies of Cefdinir

Flow Rate	Tailing Factor	Peak Area			SD	% RSD
		Trial 1	Trial 2	Trial 3		
1.1 ml/min	1.79	53198.31	53221.23	53188.29	16.88	0.0317
1.3 ml/min	1.92	56341.27	56432.63	56448.74	57.95	0.1027
1.5 ml/min	2.08	59981.24	60112.45	59895.14	109.43	0.1823
Average. of %RSD = 0.1055%						

Table No. 8.7: Summary of validation parameters of Cefdinir

Sr. No.	Parameter	Result
1.	Linearity indicated by correlation coefficient	0.997
2.	Linear regression equation	y = 2536x + 1988
3.	Range	4-24 µg/ml
4.	Interday Precision (%RSD)	0.2303%
5.	Intraday Precision (%RSD)	0.3578%
6.	Limit of Detection	0.1992 µg/ml

7.	Limit of Quantification	0.6037µg/ml
8.	Robustness indicated by % RSD	0.1055%

[B] Degradation Studies[20]

The International conference on Harmonization (ICH) guidelines entitled stability testing of new drug substance and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the impurity study of the Cefdinir using the proposed method.

1. Acidic degradation

In 6 different 10 ml volumetric flask 2 ml of working standard solution (100 ppm) was mixed with 3 ml of 0.1N, 0.5N, 1N, 1.5N, 2N, 3N hydrochloric acid and kept for 1 hours. After 1 hours solution was neutralized with sodium hydroxide then solution was diluted to 10 ml with Phosphate buffer (pH- 6.8) : Acetonitrile (60:40 V/V) and injected in stabilized

chromatographic conditions. Under this condition, degradation was observed at 0.1N hydrochloric acid (Deg-1, RT= 1.476, Table No. 8.8 and Fig.8.6)

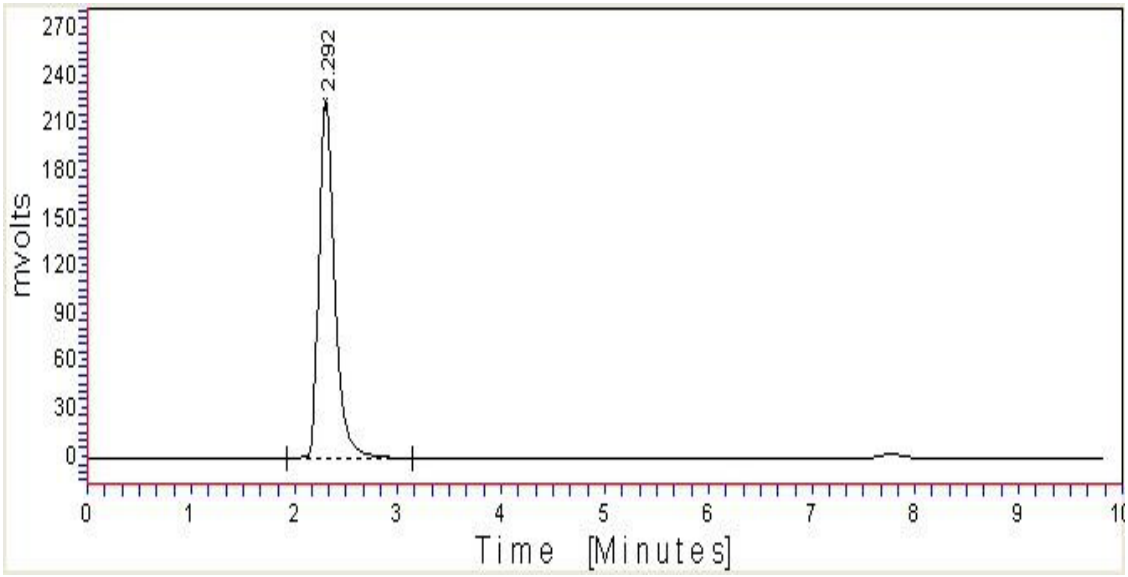

Fig. 8.5: Chromatogram of Cefdinir (20 µg/ml, RT = 2.292) with mobile phase Phosphate buffer (pH-6.8): Acetonitrile (60:40 V/V)

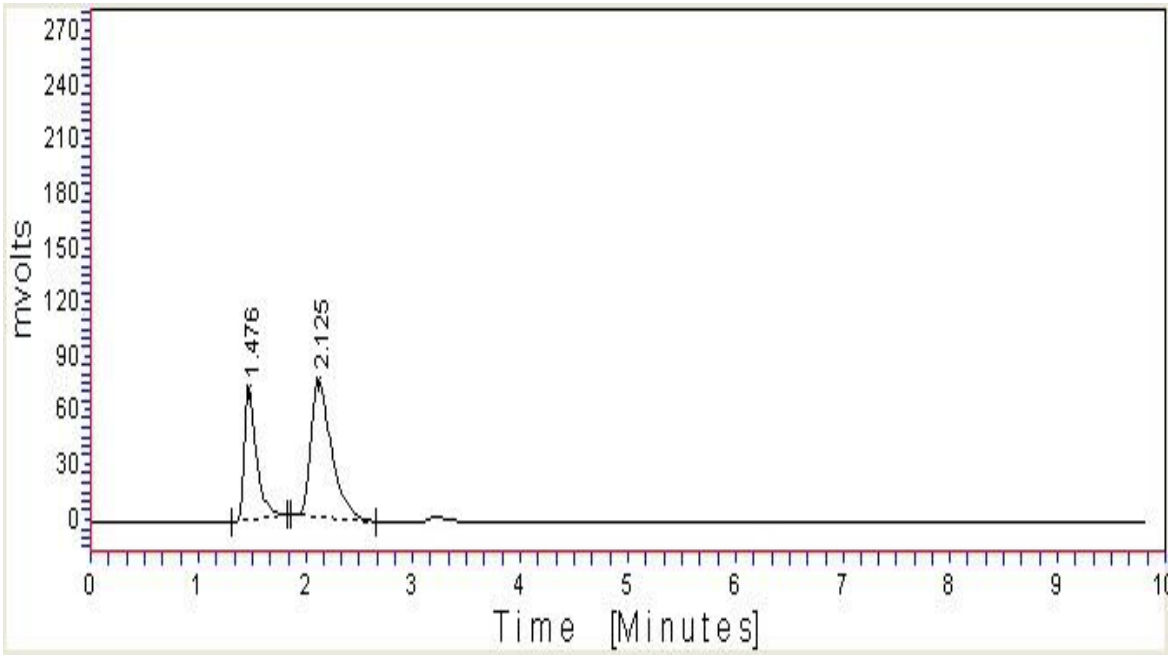

Fig. 8.6: Chromatogram of Cefdinir after acidic degradation with degradation product at RT 1.476

- **% purity of standard drug without stress condition**

Absorbance of standard solution (20 ppm) at 287 nm = 50113.32

Amount of drug present in standard solution was x

From regression equation were finding out drug content of 20 ppm solution i. e.

$$Y = m x + c$$

Where, Y = Absorbance; m = Slope

x = Concentration c = Constant

$$Y= 2536x + 1988$$

$$50113.32 = 2536x + 1988$$

$$X= 18.97 \mu g/ml$$

The drug present in 20 ppm solution is 18.97 µg/ml

% drug content in given solution was calculated as,

20 µg /ml corresponds to 100%, so 18.97 µg /ml corresponds to A%

$$A= 18.97 \times 100/ 20$$

$$A = 94.88\%$$

The % drug content in standard solution of 20 ppm was found to be 94.88%

- **% purity of drug after acidic condition**

Absorbance of acid degraded sample (20 ppm) at 287 nm= 42365.23

Amount of drug present in acid degraded solution was x

From regression equation were finding out drug content of degraded sample of 20 ppm solution i. e.

$$Y = m x + c$$

Where, Y = Absorbance; m = Slope

x = Concentration c = Constant

$$Y= 2536x + 1988$$

$$42365.23= 2536x + 1988$$

$$X=15.92 \mu g /ml.$$

The drug present in degraded 20 ppm solution was **15.92 µg /ml**.

% Drug content in given degraded solution was calculated as,

20 µg /ml corresponds to 100%, so 15.92 µg /ml corresponds to B%

B= 15.92 x 100/20

B= 79.60%

The % drug content in degraded solution of 20 ppm was found to be 79.60%

The difference between the percent drug content of standard solution and percent drug content of degraded solution gives the percentage of drug degraded.

94.88-79.60=15.28%

Therefore, **15.28 % drug has been degraded in acidic condition.**

The difference between the amount of drug present in standard solution and the amount of drug present in degraded solution gives the amount of drug degraded.

18.97-15.92=3.05 µg/ml

3.05 µg/ml of drug have been degraded in acidic condition.

2. Alkaline degradation

In 6 different 10 ml volumetric flask 2 ml of working standard solution (100 ppm) was mixed with 3 ml of 0.1N, 1N, 1.5N, 2N, 2.5N, 3N sodium hydroxide respectively and kept for 1 hours. After 1 hours solution was neutralized with dilute hydrochloric acid then solution was diluted to 10 ml with Phosphate buffer (pH-6.8) : Acetonitrile (60:40 V/V) and injected & degradation was observed at 0.1N sodium hydroxide.(Deg-2, RT = 1.46, Table No. 8.8 and Fig. 8.7).

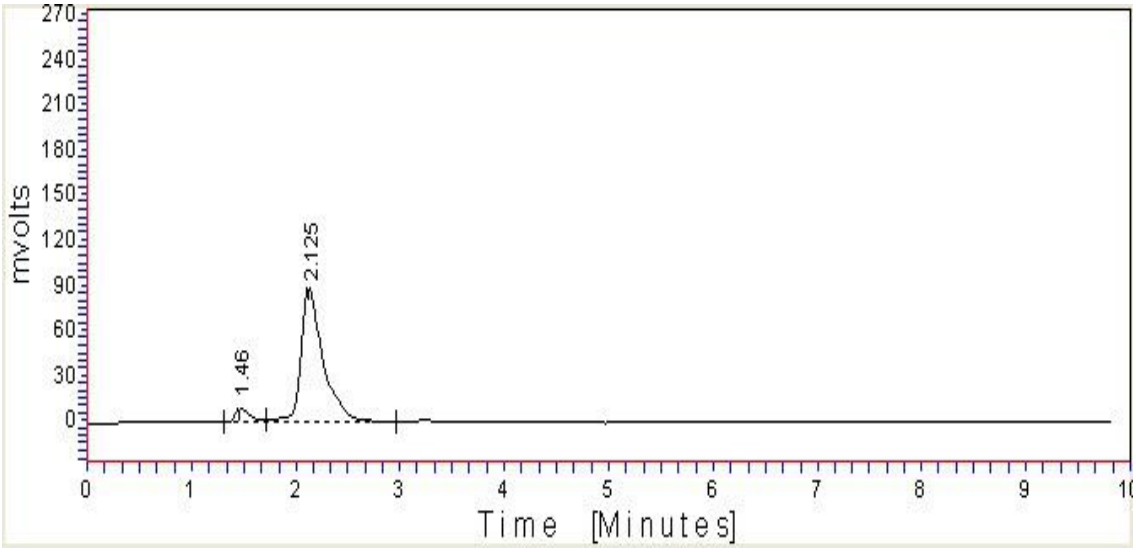

Fig. 8.7: Chromatogram of Cefdinir after alkaline degradation with degradation product at RT 1.46

- **% purity of standard drug without stress condition**

The drug present in 20 ppm solution is **18.97 µg/ml**

The % drug content in standard solution of 20 ppm was found to be **94.88%.**

- **% purity of drug after Alkaline condition**

Absorbance of alkaline degraded sample (20 ppm) at 287 nm= 46581.12

Amount of drug present in alkaline degraded solution was x

From regression equation were finding out drug content of degraded sample of 20 ppm solution i. e.

$$Y = m x + c$$

Where, Y = Absorbance; m = Slope

x = Concentration c = Constant

$$Y= 2536x + 1988$$

$$46581.12= 2536x + 1988$$

$$X=17.58 \text{ µg/ml.}$$

The drug present in degraded 20 ppm solution was **17.58 µg /ml.**

% Drug content in given degraded solution was calculated as,

20 µg /ml corresponds to 100%, so 17.58 µg /ml corresponds to B%

B= 17.58 x 100/20

B= 87.92%

The % drug content in degraded solution of 20 ppm was found to be **87.92%**

The difference between the percent drug content of standard solution and percent drug content of degraded solution gives the percentage of drug degraded.

94.88-87.92= 6.96%

Therefore, **6.96% drug has been degraded in alkaline condition.**

The difference between the amount of drug present in standard solution and the amount of drug present in degraded solution gives the amount of drug degraded.

18.97-17.58=1.39 µg/ml

1.39 µg/ml of drug have been degraded in alkaline condition.

3. Photo-degradation studies

The photochemical stability of the drug was studied by exposing the drug sample to UV light for 24 hour 10 mg after exposure, accurately weighed 10 mg of drug in 100 ml of Phosphate buffer to get concentration 100 µg/ml. 2 ml standard stock solution of Cefdinir was then diluted in Phosphate buffer (pH-6.8) : Acetonitrile (60:40 V/V) up to 10 ml to get working standard solution 20 µg/ml and was then injected in stabilized chromatographic conditions. (Deg- 3, RT- 2.255, Table No. 8.8 and Fig. 8.8)

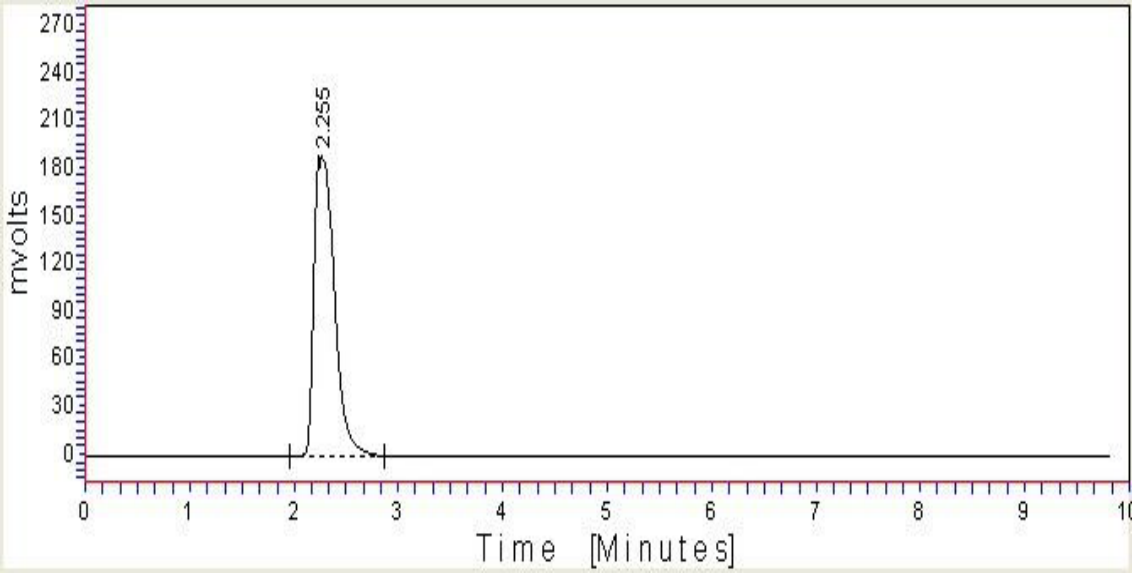

Fig. 8.8: Chromatogram of Cefdinir after photolytic degradation with degradation product at RT 2.255

- **% purity of standard drug without stress condition**

The drug present in 20 ppm solution is **18.97 µg/ml**

The % drug content in standard solution of 20 ppm was found to be **94.88%.**

- **% purity of drug after photolytic condition**

Absorbance of photolytic degraded sample (20 ppm) at 287 nm= 49223.26

Amount of drug present in photolytic degraded solution was x

From regression equation were finding out drug content of degraded sample of 20 ppm solution i. e.

$$Y = m x + c$$

Where, Y = Absorbance; m = Slope

x = Concentration c = Constant

$$Y= 2536x + 1988$$

$$49223.26= 2536x + 1988$$

$$X=18.62 \text{ µg/ml.}$$

The drug present in degraded 20 ppm solution was 18.62 µg/ml.

% Drug content in given degraded solution was calculated as,

20 µg/ml corresponds to 100%, so 18.62 µg/ml corresponds to B%

$$B = 18.62 \times 100/20$$

$$B = 93.10\%$$

The % drug content in degraded solution of 20 ppm was found to be **93.10%**

The difference between the percent drug content of standard solution and percent drug content of degraded solution gives the percentage of drug degraded.

$$94.88-93.10=1.78\%$$

Therefore, **1.78% drug has been degraded in photolytic condition.**

The difference between the amount of drug present in standard solution and the amount of drug present in degraded solution gives the amount of drug degraded.

$$18.97-18.62=0.35 \text{ µg/ml}$$

0.35 µg/ml of drug have been degraded in photolytic condition.

4. Thermal degradation

Thermal studies were performed by keeping drug sample in oven (70ºC) for a period of 48 hours. 10 mg of exposed drug was weighed accurately and transferred to a 100 ml of volumetric flask and dissolve in 100 ml Phosphate buffer. From this 20 ppm solution was prepared and the solution then injected in stabilized chromatographic conditions. (Deg- 4, RT- 2.263 Table No. 8.8 and Fig. 8.9)

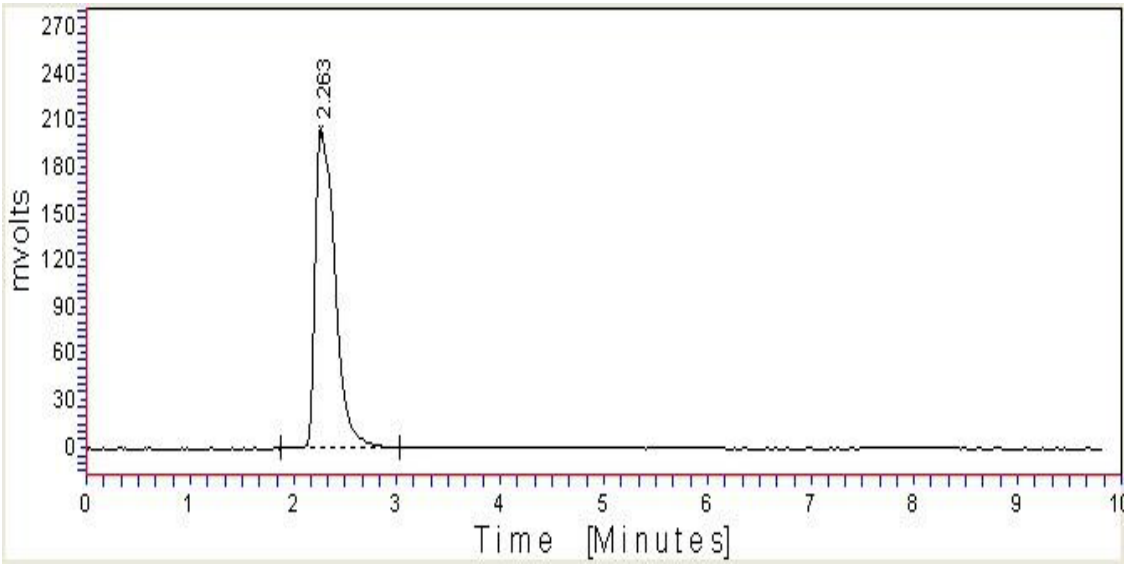

Fig. 8.9: Chromatogram of Cefdinir after thermal degradation with degradation product at RT 2.263

- **% purity of standard drug without stress condition**

The drug present in 20 ppm solution is **18.97 µg/ml**

The % drug content in standard solution of 20 ppm was found to be **94.88%.**

- **% purity of drug after thermal condition**

Absorbance of thermal degraded sample (20 ppm) at 287 nm= 49988.34

Amount of drug present in thermal degraded solution was x

From regression equation were finding out drug content of degraded sample of 20 ppm solution i. e.

$$Y = m x + c$$

Where, Y = Absorbance; m = Slope

x = Concentration c = Constant

$$Y= 2536x + 1988$$

$$49988.34= 2536x + 1988$$

$$X=18.92 \text{ µg/ml.}$$

The drug present in degraded 20 ppm solution was **18.92 µg/ml.**

% Drug content in given degraded solution was calculated as,

20 µg /ml corresponds to 100%, so 18.92 µg /ml corresponds to B%

B = 18.92 x 100/20

B = 94.63%

The % drug content in degraded solution of 20 ppm was found to be **94.63%**

The difference between the percent drug content of standard solution and percent drug content of degraded solution gives the percentage of drug degraded.

94.88-94.63=0.25%

Therefore, **0.25% drug has been degraded in thermal condition.**

The difference between the amount of drug present in standard solution and the amount of drug present in degraded solution gives the amount of drug degraded

18.97-18.92=0.05 µg/ml

0.05 µg/ml of drug have been degraded in thermal condition.

5. **Oxidative study**

   In 5 different volumetric flask 2 ml of working standard solution was mixed with 3 ml 3%, 4%, 5%, 6%, 30% hydrogen peroxide respectively .The solution was diluted to 10 ml with Phosphate buffer (pH-6.8) : Acetonitrile (60:40 V/V) and kept for 1 hours at RT. The solution was injected in stabilized chromatographic conditions. The degradation was found at 6% hydrogen peroxide (Deg- 5, RT =2.096, Table No. 8.8 & Fig.8.10).

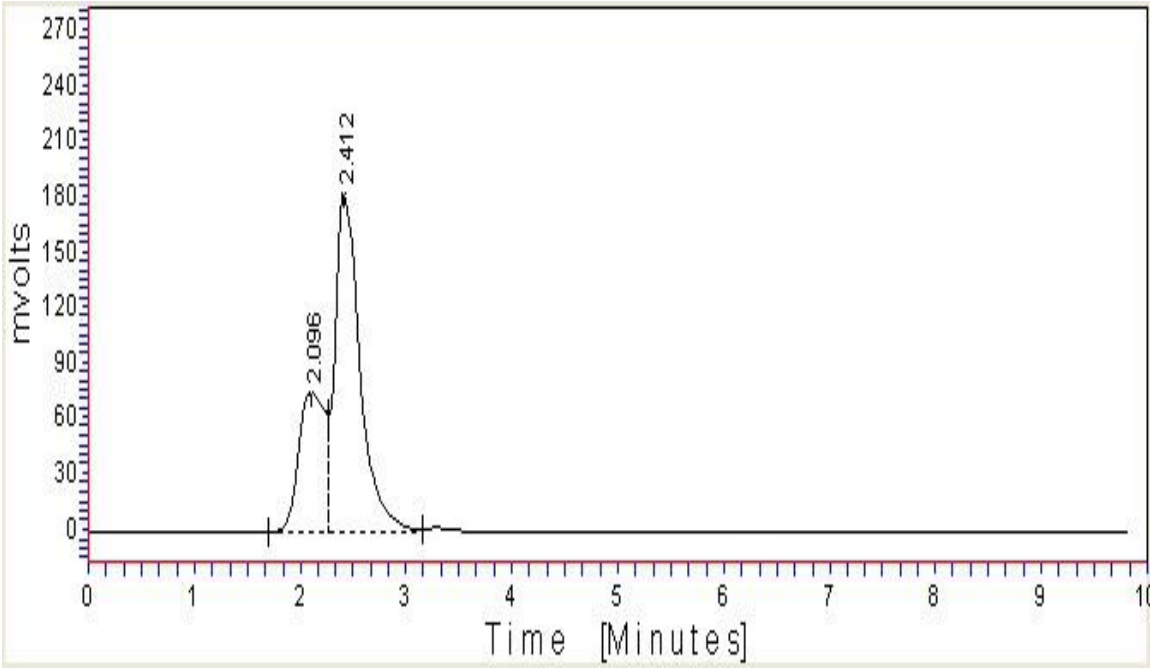

Fig. 8.10: Chromatogram of Cefdinir after oxidative degradation with degradation product at RT 2.096

- **% purity of standard drug without stress condition**

The drug present in 20 ppm solution is **18.97 µg/ml**

The % drug content in standard solution of 20 ppm was found to be **94.88%**

- **% purity of drug after oxidative condition**

Absorbance of oxidative degraded sample (20 ppm) at 287 nm= 47863.18

Amount of drug present in oxidative degraded solution was x

From regression equation were finding out drug content of degraded sample of 20 ppm solution i. e.

$$Y = m x + c$$

Where, Y = Absorbance; m = Slope

x = Concentration c = Constant

$$Y= 2536x + 1988$$

$$47863.18= 2536x + 1988$$

$$X=18.08 \mu g /ml.$$

The drug present in degraded 20 ppm solution was 18.08 µg /ml.

% Drug content in given degraded solution was calculated as,

20 µg /ml corresponds to 100%, so 18.08 µg /ml corresponds to B%

$$B = 18.08 \times 100/20$$

$$B = 90.40\%$$

The % drug content in degraded solution of 20 ppm was found to be 90.40%

The difference between the percent drug content of standard solution and percent drug content of degraded solution gives the percentage of drug degraded.

$$94.88-90.40 = 4.48\%$$

Therefore, **4.48% drug has been degraded in oxidative condition.**

The difference between the amount of drug present in standard solution and the amount of drug present in degraded solution gives the amount of drug degraded.

$$18.97-18.08=0.89 \text{ µg /ml}$$

0.89 µg /ml of drug have been degraded in oxidative condition.

Table No. 8.8: Summary of stress degradation studies of Cefdinir

Sr. No.	Stress degradation parameter	Peak area	concentration of degraded PB* (µg/ml)	% degradation	RT of degraded product
1.	Initial	50113.32	-	-	-
2.	Acid degradation	42365.23	3.05	15.28	1.476
3.	Alkali degradation	46581.12	1.39	6.96	1.46

4.	Photolytic degradation	49223.26	0.35	1.78	2.255
5.	Thermal degradation	49988.34	0.05	0.25	2.263
6.	Oxidative degradation	47863.18	0.89	4.48	2.096

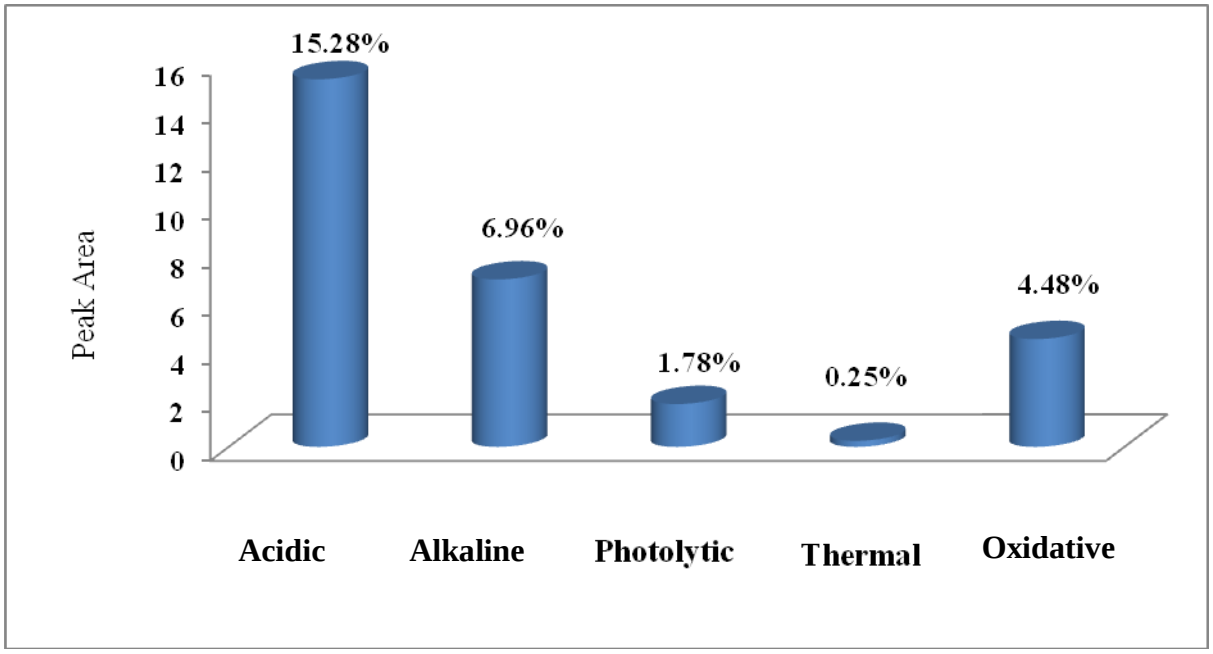

Fig. 8.11: Bar Graph Indicating Stress Degradations

Isolation of degradant by Flash Chromatography[26,27]

Flash chromatography (medium pressure chromatography):

Flash chromatography is a fast and inexpensive separation technique for the purification of organic syntheses products e.g. in drug discovery or from natural extracts. It is a popular alternative when other separation techniques cannot be used or are too difficult.

Fig. 10.1: Instrument of Flash Chromatography

Flash chromatography provides a rapid and inexpensive general method for the preparative separation of mixtures requiring only moderate resolution. It can be applied to normal-phase and reversed-phase separation. Flash chromatography can endure relatively high flow rate with low pressure, offering good separation in a short time under a proper chromatographic condition.

In flash chromatography Columns are disposable plastic cartridges, advantage of cartridges are time save and reproducibility. Based on sample volume we may select different size of cartridges. Now a day's readily prepared cartridges are available based on particle size and stationary phase volume. Flash chromatography is cost effective and low maintenance. In the case of the target molecule or compound is in high concentration, flash Chromatography is preferable. Then we may isolate the compound with high purity. In the case of sample have more chemical constituents, without information of concentrations of that chemical constituents, preparative chromatography is preferable.

Table No. 10.1: The Properties of Commonly Used Flash Solvents

Solvent	Density (g/ml)	Elution Strength	Solvent Group	Boiling Point (°C)
n-Hexane	0.66	0.01	1	69

Toluene	0.87	0.22	7	110
Dichloromethane	1.33	0.30	5	40
Ethyl Acetate	0.90	0.45	6	77
Acetone	0.79	0.53	6	56
Acetonitrile	0.78	0.50	6	82
Isopropanol	0.79	0.60	3	82
Methanol	0.79	0.70	3	65

Advantages of Flash Chromatography:

1) Maximum Quantities of the sample can be separated (0.5-2.0 g).
2) Separation time is 10-15 min.
3) Elaborate equipment and the purchase of expensive equipment are not necessary.
4) Cartridges are reusing full, nearly 8 times cheaper than Preparative columns.
5) No need to solubility of sample in Mobile phase.
6) More useful in separation of various antibiotics, Impurities, Peptides.
7) This technique saves time and solvents.
8) Reliable and cost effective.

Specifications:

1. Instrument model name : Combi Flash Campanion
2. Manufactured By :Teledyne ISCO
3. Column : Gold 80

4. Mobile phase : Chloroform :Methanol (1 :1)
5. Detection wavelength : 254 nm
6. Monitor wavelength : 287 nm
7. Flow Rate : 25 ml/min
8. Equilibration Volume : 100.0 ml
9. Rack : 16 mm x 150 mm tubes
10. Peaks Tube Volume : 13.0 ml

General Process of Flash Chromatography:

- In traditional column chromatography a sample to be purified is placed on the top of a column containing some solid support, often silica gel.
- The rest of the column is then filled with a solvent (or mixture of solvents) which then runs through the solid support under the force of gravity.
- The various components to be separated travel through the column at different rates and then can be collected separately as they emerge from the bottom of the column. Unfortunately, the rate at which the solvent percolates through the column is slow.
- In flash chromatography however air pressure is used to speed up the flow of solvent, dramatically decreasing the time needed to purify the sample, therefore making the column and running the separation could take less than 10 - 15 minutes.

Selection of mobile phase by TLC:

For the development of TLC on trial basis prepared a mobile phase consisting solvent of hexane, ethyl acetate, chloroform, methanol, toluene in proper composition. Saturation of chamber with mobile phase was done. After that applying spot of drug (conc. of 20 µg/ml) solution on precoated TLC plate and further place in mobile phase for development of TLC plate. Examined that TLC plate under UV chamber or iodine chamber. Following the results.

PCR)

Fig. 10.2: TLC plate with mobile phase Hexane: Ethyl Acetate (1:1) No Result

Fig. 10.3: TLC plate with mobile phase Chloroform: Methanol (1:1) Good Result

Evaluation of the Chromatogram:

$$\text{Retention factor} = \frac{\text{Distance travelled by the solute from the origin line}}{\text{Distance travelled by the solvent from the origin line}}$$

R.F. Value = 3.7/7

R.F. Value = 0.52

Degradation study by using Flash Chromatography:

Preparation of Standard stock solution:

100 mg of bulk drug was weighed accurately and transfer in 10 ml of volumetric flask. Drug was dissolve in methanol and volume was made up to 10 ml with same solvent. So as to get the conc. of 10,000 µg/ml.

Preparation of Degradant:

1) Acidic Degradant:

Trial Peak: 1

Procedure:

- For trial prepared a degradant of conc. of 20 µg/ml. In 10 ml volumetric flask pipette out 2 ml of working standard solution of drug from 100 ppm was mixed with 3 ml of 0.1N HCL and kept for 30 min for heating on water bath. After 30 min solution was diluted up to 10 ml with methanol.
- The (200 mg) degradant solution was adsorbed over silica gel (# 60 – 120) in the ratio 1:4 (drug to silica gel) and finally dried under vacuum below 60^0 C. A column of 5 litres capacity was first loaded with 1 to 2 g of silica gel (# 60-120) with chloroform as solvent (dry packing).
- The adsorbed material (200 mg) was charged and eluted with chloroform: methanol gradient (100:0---90:10---80:20---70:30---60:40---50:50---40:60---30:70---20:80---0:100). Fractions of 100 ml were collected. The fractions collected were concentrated by distillation under vacuum using rota vapour and weighed. Graph shown in Fig. 10.5.

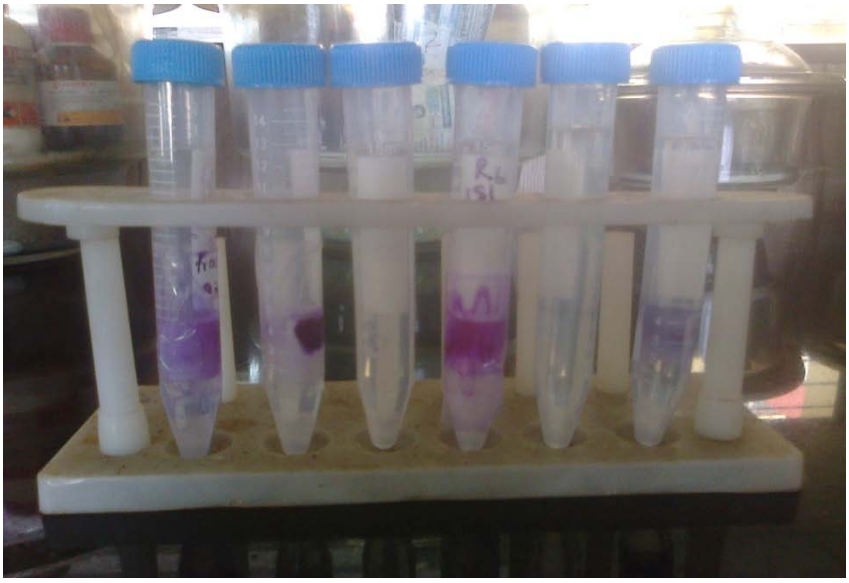

Fig. 10.4: Vials for collection of sample

Isolated fractions of acid degradant were collected in sample vials shown in fig no. 10.4. There was 6 fraction of degradant from that fraction 1 was shown good isolation of degradant. So these fractions 1 continue for further process for characterization by UV, HPLC, IR, MS, and NMR.

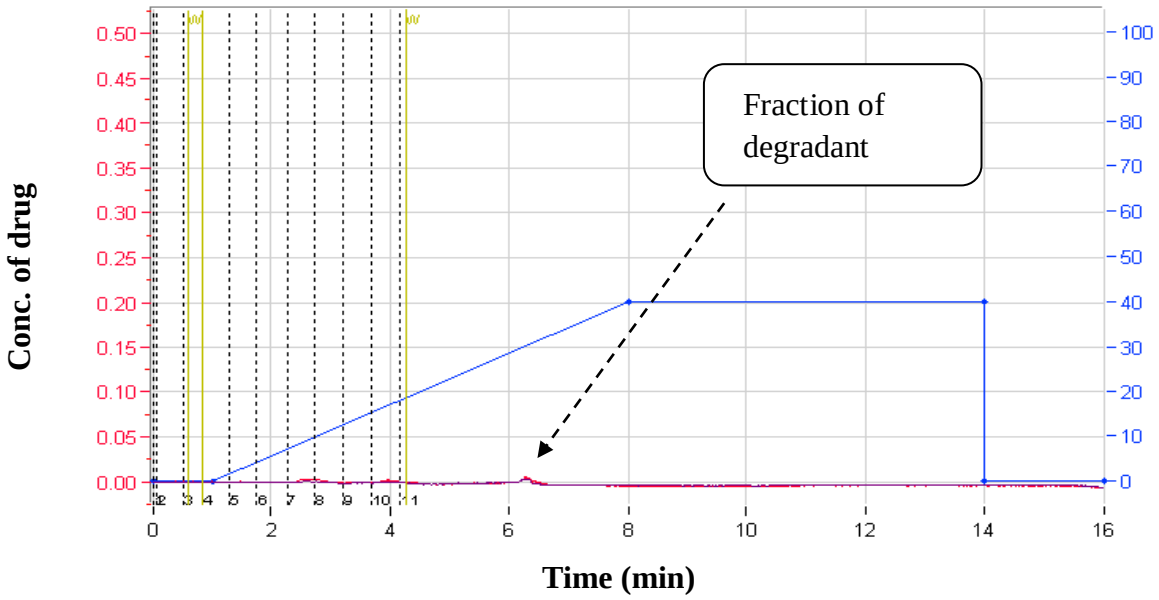

Fig. 10.5: Chromatogram of API drug (20 µg/ml) at mobile phase Chloroform: Methanol (1:1)

Peak with API Drug:

Procedure:

- Weighed of powder of API drug mixed with 1 gm of silica gel (grade 60). Properly mixed API with silica gel on petriplate with glass rod. Further place into the cartridge and injected in stabilized chromatographic condition. Graph shown in Fig. 10.6. And obtained 6 isolates of acid degradant has collected in separate tubes.

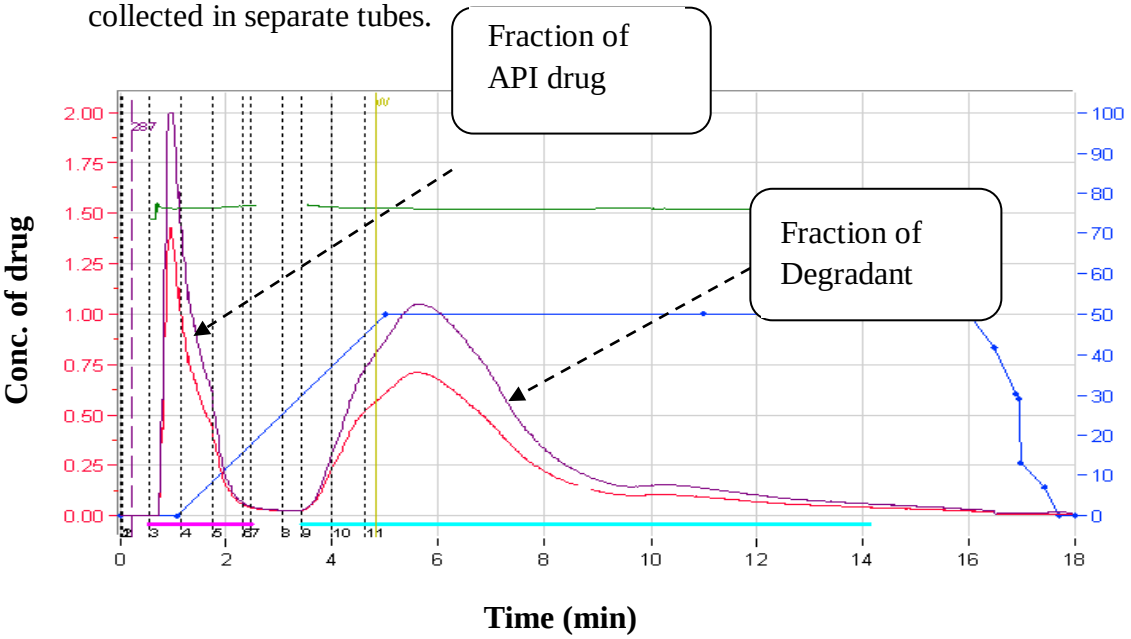

Fig. 10.6: Chromatogram of API drug and degradant at mobile phase Chloroform: Methanol (1:1)

Resulted Peak (Acid Degradant):

- Prepared a conc. of 2000 µg/ml from above working standard solution (10,000 ppm). In 10 ml of volumetric flask pipette out 0.2 ml of working standard solution was mixed with 5 ml of 0.5N HCL and kept for 30 min for heating on water bath. After 30 min solution was diluted up to 10 ml with methanol.
- The prepared (2000 ppm) degradant solution was adsorbed over silica gel (# 60 – 120) in the ratio 1:4 (drug to silica gel) and finally dried under vacuum below 60^0 C. A column of 5 litres capacity was first loaded with 1 to 2 g of silica gel (# 60-120) with chloroform as solvent (dry packing).
- The adsorbed material (200 mg) was charged and eluted with chloroform: methanol gradient (100:0---90:10---80:20---70:30---60:40---50:50---40:60---30:70---20:80---0:100). Fractions of 100 ml were collected. The fractions collected were concentrated by distillation under vacuum using rota vapour and weighed. Graph shown in Fig. 10.7.

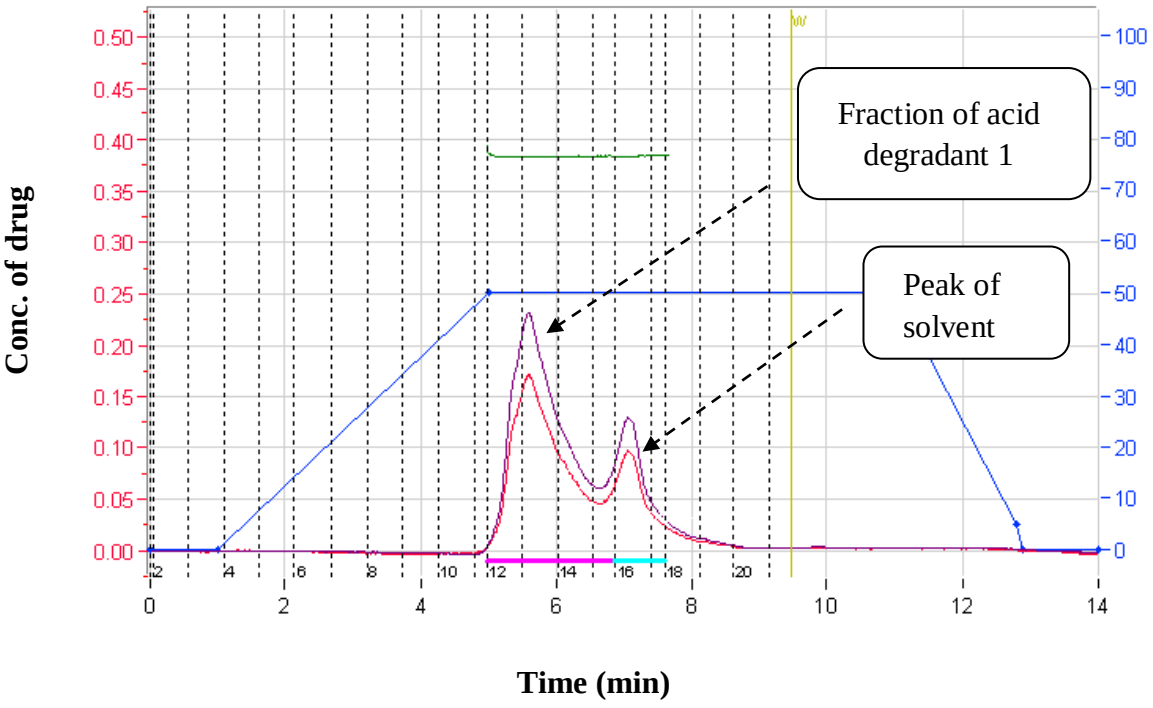

Fig. 10.7: Chromatogram of acidic degradant fraction 1 at mobile phase

Chloroform: Methanol (1:1)

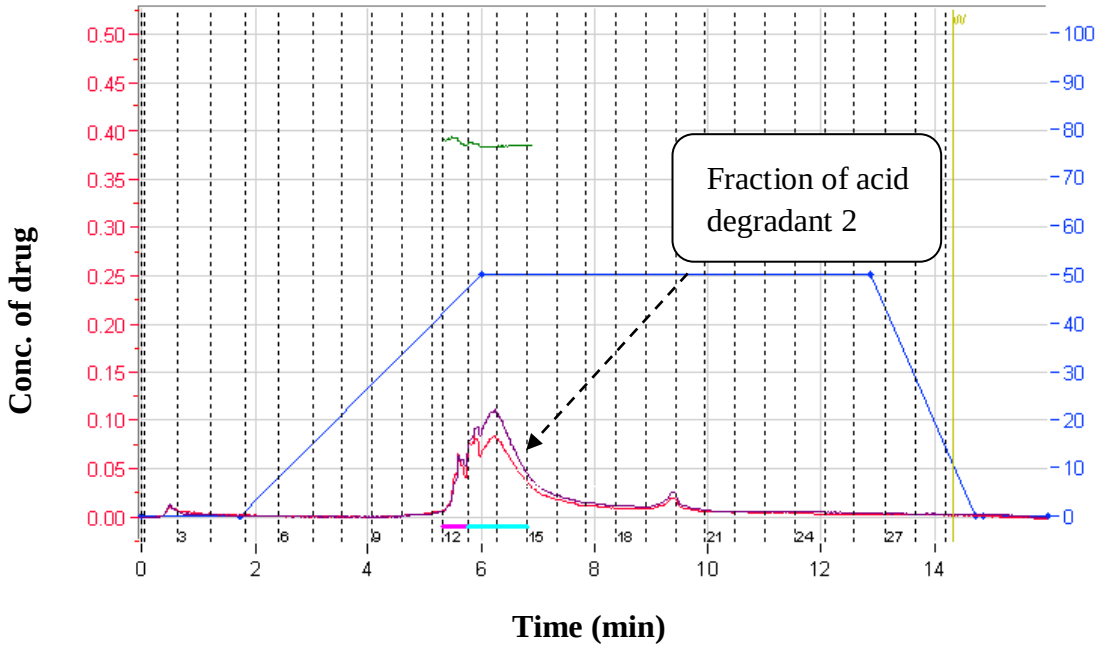

Fig. 10.8: Chromatogram of acidic degradant fraction 2 at mobile phase

Chloroform: Methanol (1:1)

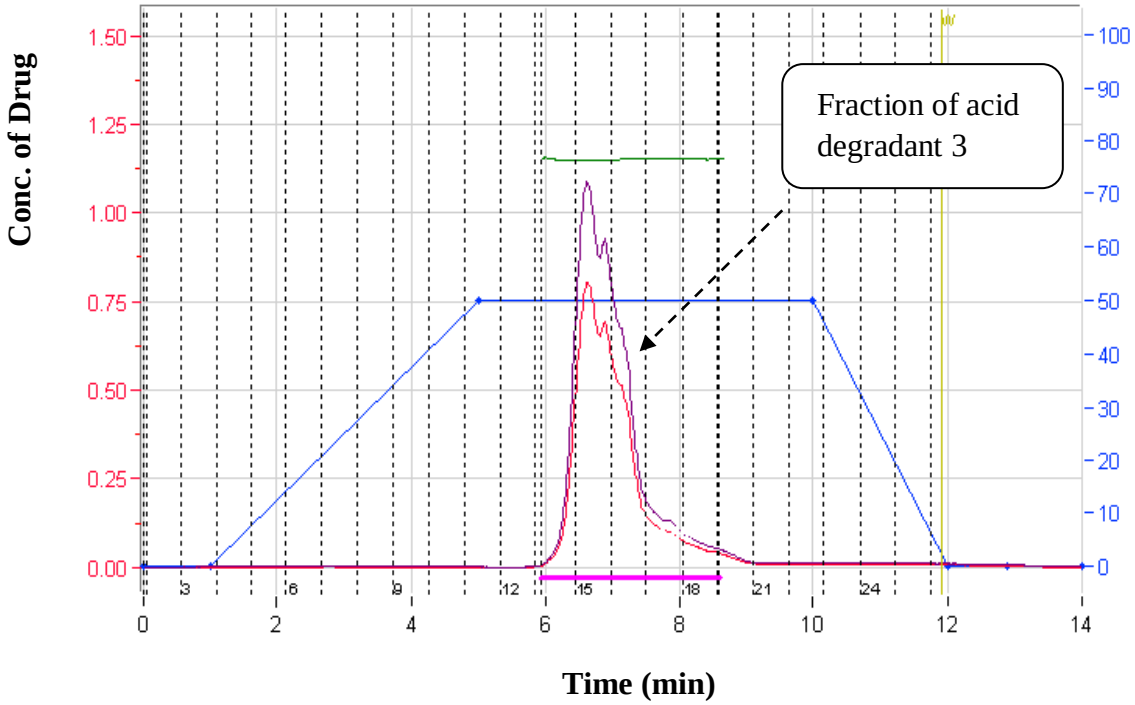

Fig. 10.9: Chromatogram of acidic degradant fraction 3 at mobile phase

Chloroform: Methanol (1:1)

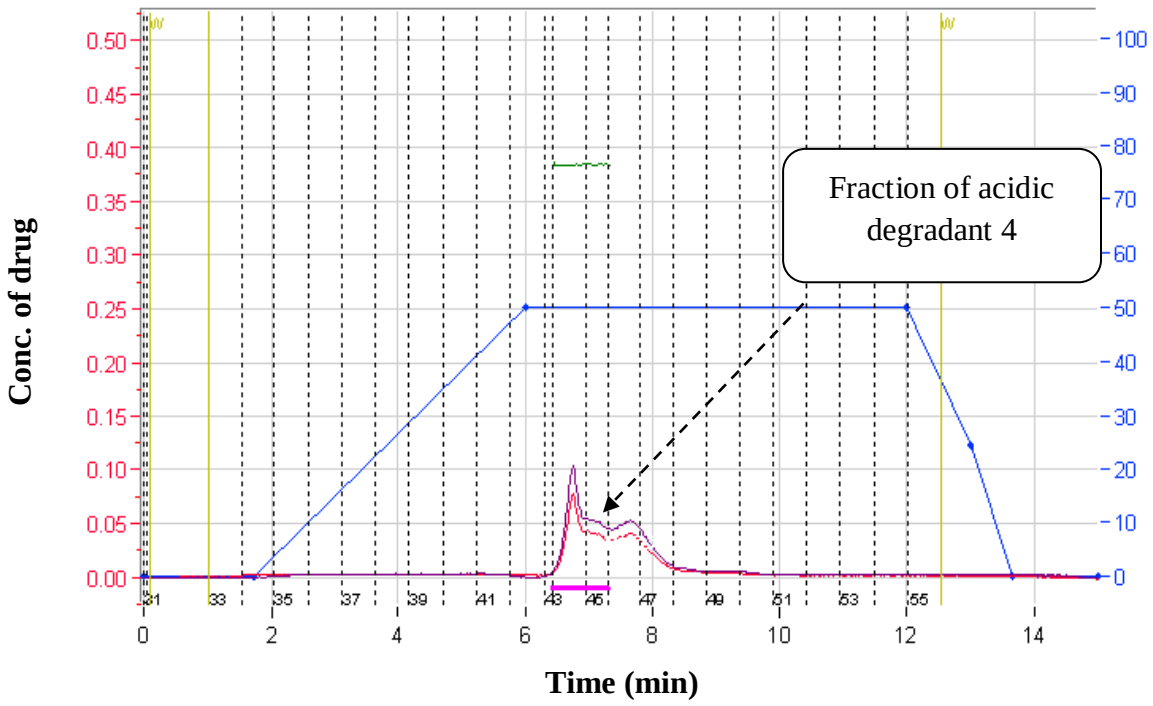

Fig. 10.10: Chromatogram of acidic degradant fraction 4 at mobile phase

Chloroform: Methanol (1:1)

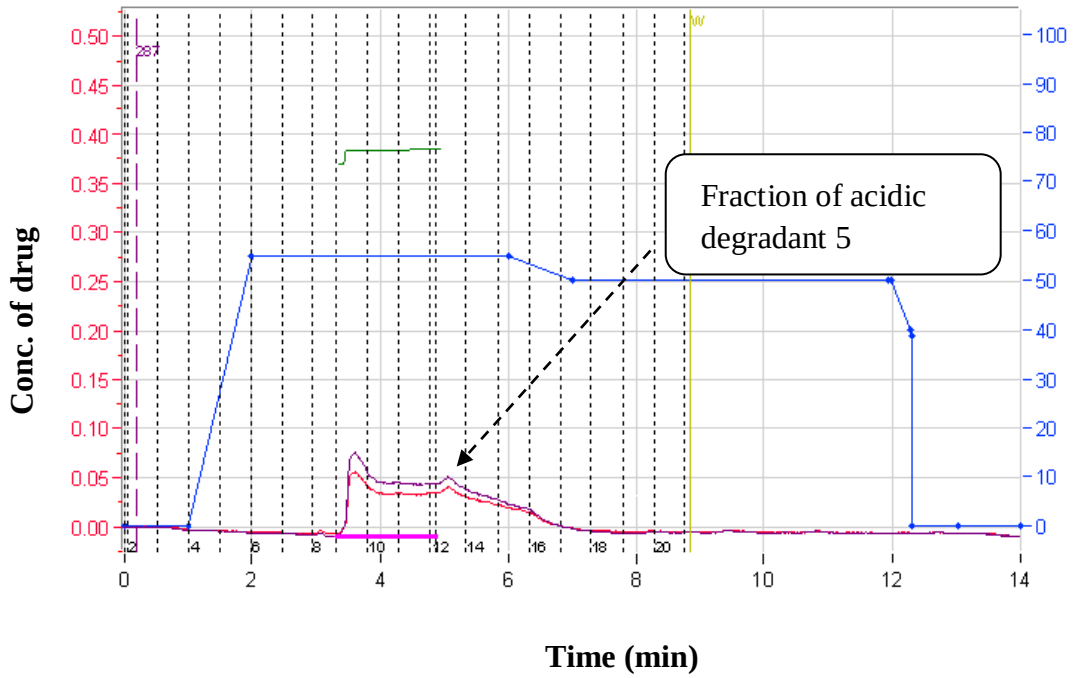

Fig. 10.11: Chromatogram of degradant fraction 5 at mobile phase

Chloroform: Methanol (1:1)

Characterization of acid degradant fraction 1:

The structures of isolated fraction of degradation products were characterized by UV, HPLC, NMR, Mass spectra and functional groups were identified by IR spectra. The spectra of API drug were compared to that of degradation product in order to ascertain the changes occurred in drug due to degradation.

1) UV Spectra-

To analyze the collected fraction of impurities obtained from flash chromatography samples was scanned under UV in the range of wavelength 200-400 nm. In followed UV spectra it shows change in wavelength that was at 268 nm. From this result it was conclude that the degradants are isolated successfully. Overlay shown in Fig. 10.12.

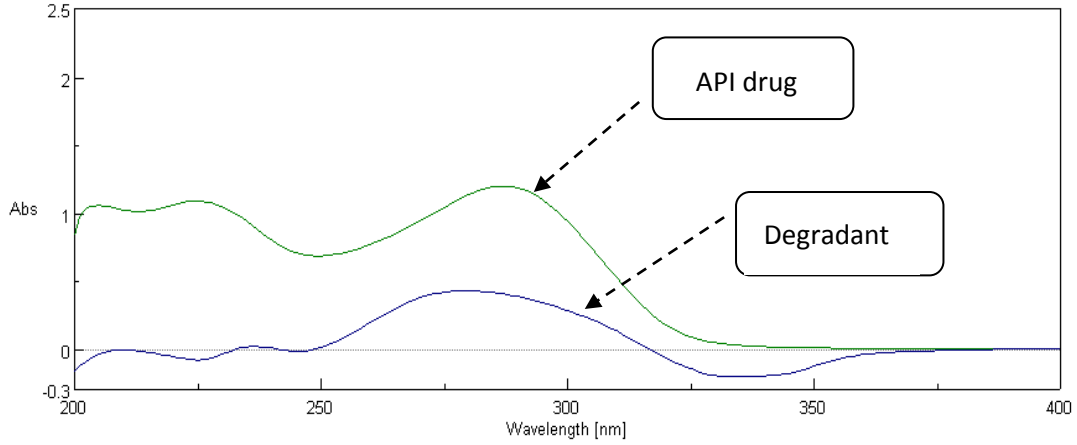

Fig. 10.12: Overlaid of API drug and acidic degradant fraction 1

2) HPLC -

To analyze the collected fraction of impurities obtained from flash chromatography samples was scanned under HPLC over the range of 200 - 400 nm by using mobile phase Water: Methanol (80:20). Degradant peak shown in fig. 10.13.

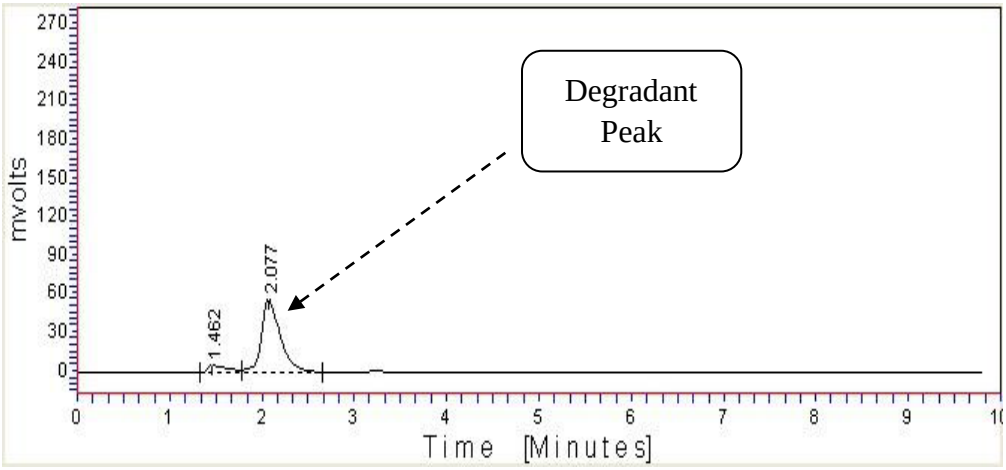

Fig. 10.13: Chromatogram of fraction of impurities with mobile phase

Water: Methanol (80: 20)

3) FT-IR:

Specification of FT-IR

Model - JASCO- M 4100 FT-IR

Preparation of sample for IR

The collected fraction of acid degradant fraction 1 adsorbed on sufficient Qty. of silica gel. This residue was then mixed with KBr in the ratio 1:300 and this sample was analyzed. The observed frequencies are shown in table no. 10.2.

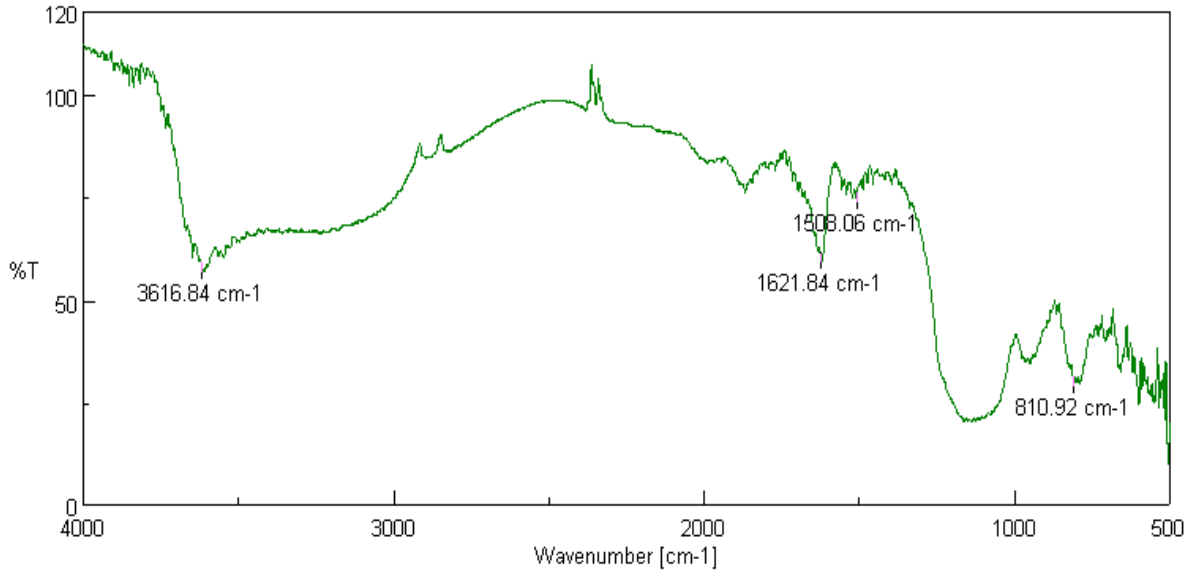

Fig. 10.14: FT-IR Spectrum of acid degradant

Table No. 10.2: IR Observed Frequency of Acid Degradant

Present group	Observed frequency (cm^{-1})
N-H, C-C stretch, C-H	810.92
N-H, O-H, C-H bending	1508.06
N-H, C=N, C=O, C-C bending	1621.84
N-H, O-H, C=O stretch	3616.84

Result

- One broad band observed at higher wave no. 3616.84 cm^{-1} indicates the presence of a hydroxyl group.
- The IR Spectrum of degraded product do not contain any peak for C=O functional group. It can be concluded that they have been convert to -OH group during the process of chlorination.

4) HR-MS:

Specification of Mass Spectrometer

Model and Specifications

- Bruker Daltonik GmbH, Germany
- Impact II UHR-ToF Mass Spectrometer System
  (Impact II Ultra-High-Resolution Time-of-Flight Mass Spectrometer)

Ionization source:

- ESI (Electron Spray Ionization)
- APCI (Atmospheric Pressure Chemical Ionization)
- Mass resolution: 50,000 FSR (Full Sensitivity Resolution)
- Mass accuracy: Sub ppm mass accuracy
- Mass range: 20 to 3500 m/z

Preparation of Sample for MS

The collected fraction of acid degradant fraction 1 further diluted with solvent methanol and analysed under ESI- ionization mass spectra. Calibrate the spectra on software Bruker Compass Data Analysis 4.2. Mass spectra of degradant were obtained shown in fig. 10.15.

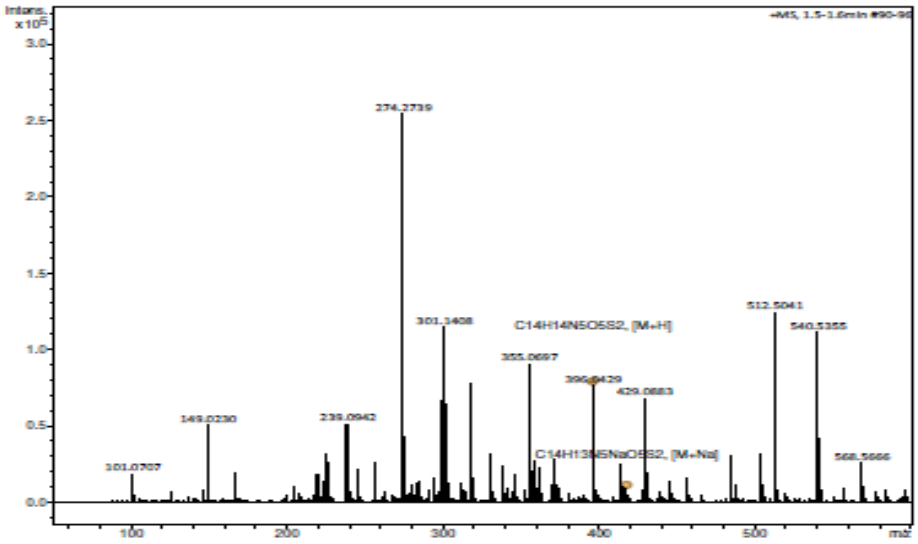

Fig. 10.15: MS spectra of acidic degradation

5) NMR:

specification of NMR Spectra

Model and Specifications

- NMR 500MHz
- Make- Bruker
- Model-Advance III HD
- Software- Topspin 3.2
- Solvent- DMSO

Preparation of sample for NMR

The collected fraction of acid degradant fraction 1 was placed in petriplate to evaporate all the solvent at RT. The obtained residue was collected and dissolved in DMSO solvent and loading on Bruker advance III HD and analysed on software Topspin 3.2 for proton and ^{13}C NMR. The spectral assignment for proton and carbon signals chemical shift values has been shown in Fig. 10.16, 10.17 resp. and table No. 10.3.

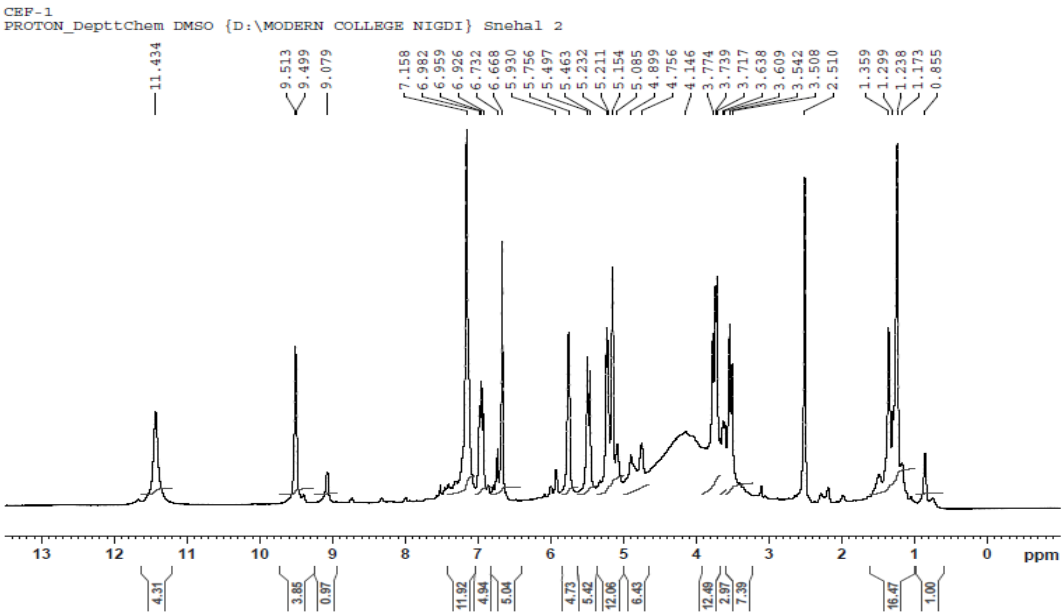

Fig. 10.16: Proton NMR spectra of acidic degradant fraction 1

Table No. 10.3: Observed Chemical shifts

Chemical Shift (ppm)	Splitting of signal
0.85	Doublet
1.29	Doublet
2.51	Singlet
3.77	Triplet
5.46	Doublet
9.51	Doublet
11.43	Singlet

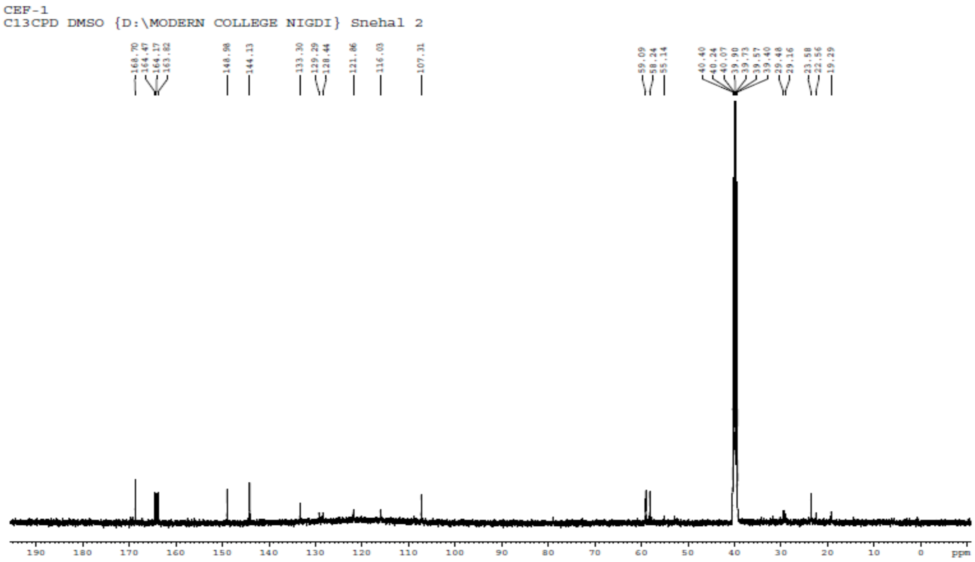

Fig. 10.17: ^{13}C NMR Spectra of acid degradant fraction 1

Observed Results

- In the ^{13}C NMR spectrum, the signals appeared at δ 164 ppm due to the thiazine hydrogen bearing carbon and attached methyl carbon.
- A signal appeared at δ 29 and 40 ppm indicating the presence of methylene group and carbonyl group or methyl hydroxy group resp.

- **UV method**

The study was discussed to develop simple, accurate and precise UV method for estimation of Cefdinir bulk drug. Standard calibration curve was plotted as absorbance vs. concentration. This straight line obeys linearity in the conc. range of 4 µg/ml to 24 µg/ml in which drug obeyed Beer-Lambert's Law. The correlation was found to be 0.998. The recovery study was carried out by preparing solution of 80%, 100% and 110% and got recovery between 93% to 102%. Precision study was carried out by three different solutions of three different conc. at three different intervals of day time. % RSD of precision studies was found to be 1.7% for intraday and 1.005% for interday. Robustness of the method was determined by carrying out the analysis under different temperature conditions i.e. at ambient temperature and at 10°C. The % RSD was found to be 0.326%. Ruggedness of the method was determined by carrying out the analysis by different analyst. % RSD was found to be 0.545%. LOD and LOQ were calculated from standard deviation and slope obtained value 0.039 ppm and 0.120 ppm respectively. From the above it was concluded that, this method was more accurate, precise,

and validated as the linearity followed the Beer-Lambert's Law. Precision robustness ruggedness % RSD was found to be less than 2% and LOD and LOQ were found to be in range.

Degradation study of API drug by UV method were carried out by placing the drug in different stress conditions. The acidic degradation, alkaline degradation, oxidative degradation, dry heat and photo degradation were found to be 15.31%, 11.04%, 1.87%, 2.6% and 1.25% respectively. Changes in λ max of acidic and alkaline degradant.

- **HPLC method**

The study was discussed to develop simple, accurate and precise UV method for estimation of Cefdinir bulk drug. The mobile phase was prepared mixing Phosphate Buffer (pH-6.8): Acetonitrile (60:40 v/v) and RT was found at 2.29 min. This straight line obeys linearity in the conc. range of 4 µg/ml to 24 µg/ml in which drug obeyed Beer-Lambert's Law. The correlation was found to be 0.997. The recovery study was carried out by preparing solution of 80%, 100% and 120% and gets recovery between 84% to 106%. Precision study was carried out by three different solutions of three different conc. at three different intervals of day time. % RSD of precision studies was found to be 0.3578% for intraday and 0.2303% for interday. Robustness of the method was determined by carrying out the analysis under different temperature conditions i.e. at ambient temperature and at ˚C8 The % RSD was found to be 0.1055%. LOD and LOQ were calculated from standard deviation and slope obtained value 0.1992 ppm and 0.6037 ppm respectively. From the above it was concluded that, this method was more accurate, precise, and validated as the linearity followed the Beer-Lambert's Law. Precision, robustness, ruggedness, %RSD was found to be less than 2% and LOD and LOQ were found to be in range.

Degradation study of API drug by HPLC method were carried out by placing the drug in different stress conditions. The acidic degradation, alkaline degradation, oxidative degradation, dry heat and photo degradation were found to be 15.28%, 6.96%, 4.48%, 0.25% and 1.78% respectively. The change in peak area was observed.

- **HPTLC Method**

The estimation of different of CEF bulk was carried out by HPTLC using mobile phase having the composition of Diethyl ether: methanol: water (8.5:1:0.5 v/v/v) on the basis of polarity of drug. Standard calibration curve was plotted as peak area vs. concentration. The

straight line obeyed linearity in the conc. range 200-600 ng/band. The correlation was found to be 0.997. The % recovery was found to be 97-100%. The method is found to be precise as %RSD of intraday and interday precision were less than 2%. Robustness was carried out by changing conc. of mobile phase and by changing time from application to development and %RSD was found to be 0.0292% and 0.0293%. LOD and LOQ were found to be 0.354 ng/band and 1.073 ng/band.

Degradation study of API drug by HPTLC method were carried out by placing the drug in different stress conditions. The acidic degradation, alkaline degradation, oxidative degradation, dry heat and photo degradation were found to be 11.94%, 8.69%, 6.32%, 1.79% and 4.05% respectively. The change in peak area was observed.

- **CHARACTERIZATION TECHNIQUES**

➢ **UV**

UV spectra of both, the API drug and the acid degradant was taken in order to distinguish and by analysing overlaid spectra API drug shows peak at λ max 287 nm and acid degradant shows changes in λ max at 268 nm. It concludes that degradation was done successfully.

➢ **HPLC**

The collected fraction of impurities of acidic degradant was analyzed by using mobile phase Water: Methanol (80:20). There is isolation of degradant was done successfully.

➢ **FT-IR**

IR spectra of both, the API drug and the acid degradant were taken in order to distinguish and identify the possible structure of the unknown degradant that has formed.

- One broad band observed at higher wave no. 3616.84 cm^{-1} indicates the presence of a hydroxyl group.
- The IR Spectrum of degraded product do not contain any peak for C=O functional group. It can be concluded that they have been convert to -OH group during the process of chlorination.

➢ **MASS SPECTROSCOPY**

The MS study of API drug and its acidic degradation was performed.

The (M+1) peak was obtained at 395.41 m/z which confirm molecular weight of API drug at 396.04 m/z.

Acidic Degradation spectra of API drug were obtained. The peaks were obtained at 274.27, 149.02, 239.09, 395, 429.08. All these peaks obtained were further characterized and probable structure of each peak was estimated.

NMR SPECTROSCOPY

The ^{1}H NMR and ^{13}C NMR spectra were recorded using DMSO as a solvent. The spectral assignment for proton and carbon signals chemical shift values were obtained.

- In the ^{13}C NMR spectrum, the signals appeared at δ 164 ppm due to the thiazine hydrogen bearing carbon and attached methyl carbon.
- A signal appeared at δ 29 and 40 ppm indicating the presence of methylene group and carbonyl group or methyl hydroxy group resp.

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